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Processes in Cell Ensembles: Correlated Fluctuations and their Effects

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(Received 14 May 1960)

SUMMARY

In the study of biological systems kinetic problems arise which are not significant in the kinetic systems of interest to physical chemists. One of these biological problems is the influence of correlated fluctuations in molecular properties on the macroscopic variables of the cell. It is demonstrated that, for an ensemble consisting of systems each of which includes two chemical compounds, there is a definite effect which originates in correlated fluctuations and is reflected in a broadened distribution of the concentration of chemical constituents. It is shown that a periodically varying temperature narrows this distribution. The narrowing is a function of the frequency at which the temperature is varying.

INTRODUCTION

This study is motivated by a number of observations recorded in the literature concerning the response of cell populations to temperature variation (Zeuthen, 1958; Scherbaum, 1960). By measuring any given property of any cell in a colony, one finds that the value of that property varies from cell to cell. Rahn (1932) attempted to explain these variations by assuming a step-wise growth of the chromosomes and by attributing equal probabilities for the addition of each successive gene. He then used this treatment to investigate the dependence of the spread of division time on the number of genes. Kendall (1948, 1952) investigated the role of variable generation time in the development of a stochastic birth process. Kendall defines a 'multiple-phase birth process' which holds that duplication of a cell follows after the cell has passed through a finite number of phases. Both Rahn's model and Kendall's model lead to the conclusion that individual generation times are independent of one another. This lack of correlation of the generation times is implicit in the models.

Differences frequently exist in the properties of cells in taxonomically homogeneous colonies. A possible cause is the statistical fluctuations in the concentrations of important constituents of which each cell contains only very few molecules. We will not consider such cases in the present study.

There is no detailed experimental study of induced synchrony by a cyclic variation of temperature. The limited studies available indicate that such a procedure sometimes leads to synchrony and sometimes does not. Synchrony and spread in generation time, though perhaps interdependent in the case of a cell, are two different and distinguishable things.

Correlated fluctuations in the velocity of reactions mediated by enzymes

Klotz and his co-workers (1957, 1958) have advanced the view that macromolecules are surrounded by a sheath of 'frozen water' which must be penetrated for the molecule to diffuse towards an active enzyme site. Klotz showed that if a molecule A diffuses through this sheath to the surface it must have induced some protein denaturation near the adsorption site. When molecule A undergoes some reaction on the surface of the enzyme it will diffuse from the site, but the succeeding molecule A' diffusing to the surface will find this portion of the surface somewhat affected by the previous event. The condition of the surface is determined by random events and it is this condition which determines the reaction rate constant. Since these surface changes require a finite time, an event on the surface may influence succeeding events. Such a function of time is considered stochastic (Kittel, 1958; Uhlenbeck & Ormstein, 1954). The statistical fluctuations in the number of hydrogen bonds lead to fluctuations in both the entropy and the energy of activation for enzyme-controlled reactions.

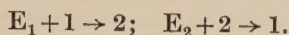
In order to have correlated events, the velocity of reaction $K(t+\tau)$ associated with an enzyme E at time $(t+\tau)$ must be related to the velocity of reaction $K(t)$ at time t . This is possible when a reacting molecule diffuses to the reaction site and completes its chemical transformation in a time roughly equal to the correlation time of the function $K(t)$. Consider an enzyme E on whose surface the compounds A and B react at two different sites. If there is energy or charge transfer between the reacting sites (cf. Szent-Gyorgyi, 1957) then these reactions are coupled. The rate of one reaction is then controlled by the rate of the other reaction and the condition of the surface. These processes are stochastic in character and lead to correlated fluctuations. Our model of the cell is then one where thousands of chemical reactions are taking place, each of which is influenced by its environment in the manner discussed above. Every so often the cell duplicates itself and breaks up into two new cells. Since it is the cumulative effect of all of these reactions that leads to cell division it is clear that any fluctuations in these reactions will be reflected in the cell division time. That is, the cells of a population will not all have the same generation time (time between two divisions).

In this study we wish to demonstrate the role of correlated fluctuations in dephasing synchronized colonies and to show how a cyclic variation in temperature 'damps' such correlation effects. The total effect of temperature variation (specially when T is large) is more complex than is assumed here. A current study (by author, to be published) analyses these phenomena.

The treatment of a simple model which displays correlated fluctuations

Constant temperature. To demonstrate the validity of our model we have to write the equations which describe the space-time variations of all the chemical species in a cell, allow for diffusion of material across the cell wall, then let the rate constants vary in a stochastic manner and show that the cumulative effect of these correlated fluctuations in the rate constants leads to a broadening in the spectral distribution of various cell properties. At present it is impossible to carry out such a complete programme. Instead, in the following sections we shall analyse the simplest example of such a kinetic system, namely, an ensemble of systems each of which is

composed of two compounds (1, 2), each of which is changed into the other pseudo-unimolecularly:



We shall assume that these processes are controlled by two different enzymes and that because of environmental effects, the rate constants are time dependent and stochastically correlated.

We shall compute the first and second moments of the distribution of the number of molecules of each compound in the ensemble. This is simpler than finding the complete distribution function for each compound over the whole ensemble. The second moment shows the 'broadening' in the distribution function due to these random variations, in whose absence there would be no dispersion. When the temperature is made to vary periodically with time the distribution narrows; i.e. the second moment is diminished.

The two compounds are now allowed to be transformed pseudo-unimolecularly into each other at the rates $k_{12}(t)$, $k_{21}(t)$, where the $k(t)$'s are stochastic variables dependent on a catalytic process. Let p_1 , p_2 be the mole fractions of each species; we have then

$$dp_1/dt = k_{12} p_2 - k_{21} p_1, \quad (1)$$

$$dp_2/dt = k_{21} p_1 - k_{12} p_2. \quad (2)$$

Define

$$p_1 + p_2 = 1, \quad (3)$$

$$k_{12} + k_{21} = \lambda. \quad (4)$$

We now analyse the rate constants into Fourier series:

$$k_{12}(t) = k_{12}^0 + \sum_n (A_n \sin nt + B_n \cos nt), \quad (5)$$

$$k_{21}(t) = k_{21}^0 + \sum_n (C_n \sin nt + D_n \cos nt), \quad (6)$$

where k_{12}^0 and k_{21}^0 are average values of the rate constants and (A_n, B_n, C_n, D_n) are all randomly distributed functions. We now assume that the random variable components average to zero within the time interval $0 \rightarrow t$ of interest to us, but not within times of the order of milliseconds. This means that a molecule could diffuse to a catalytic surface and depart from such a location within a time interval short compared to the duration of our experiment or to the rate of variation of the condition of the catalytic surface. This assumption simplifies our problem considerably, since now we can set

$$1/t \int_0^t \lambda dt = k_{12}^0 + k_{21}^0 = \lambda^0, \quad (7)$$

and our solution becomes:

$$p_1(t) = p_1(0) e^{-\lambda^0 t} + e^{-\lambda^0 t} \int_0^t k_{12} e^{\lambda^0 t} dt. \quad (8)$$

Let us now average over the ensemble A.

$$\therefore \langle p_1 \rangle_A = \langle p_1(0) \rangle_A e^{-\lambda^0 t} + e^{-\lambda^0 t} \int_0^t e^{\lambda^0 t} \langle k_{12} \rangle_A dt, \quad (9)$$

but

$$\langle k_{12} \rangle_A = k_{12}^0. \quad (10)$$

Since ensemble averages and time averages may be assumed to lead to the same results,

$$\lim_{t \rightarrow \infty} \langle p_1 \rangle_A = k_{12}^0 / \lambda^0. \quad (11)$$

Now, instead of specifying the random variables (A_n, B_n, C_n, D_n) in more detail (such as a set of Gaussianly distributed functions) we follow Uhlenbeck & Ormstein's (1954) procedure and simply seek the second moment about the origin $\langle p_1^2 \rangle_A$.

On squaring the solution for $p_1(t)$ and averaging over the ensemble A, we find

$$\langle p_1^2 \rangle_A = \langle p_1^2(0) \rangle_A e^{-2\lambda^0 t} + e^{-2\lambda^0 t} \int \int e^{\lambda(\xi+\eta)} \langle k_{12}(\xi) k_{12}(\eta) \rangle_A d\xi d\eta. \quad (12)$$

One then finds

$$\begin{aligned} \langle p_1^2 \rangle_A = \langle p_1^2(0) \rangle_A e^{-2\lambda^0 t} + \frac{(k_{12}^0)^2}{2\lambda^0 \tau_1} (1 - e^{-2\lambda^0 t}) \\ + \left(\frac{k_{12}^0}{\lambda^0} \right)^2 (1 - e^{-\lambda^0 t}) - \left(\frac{k_{12}^0}{\lambda^0} \right) (1 - e^{-\lambda^0 t}) e^{-\lambda^0 t} \end{aligned} \quad (13)$$

and

$$\tau_1^{-1} = \int_{-\infty}^{\infty} \phi(w) dw, \quad (14)$$

where $\phi(w)$ is the correlation function, where $w = \xi - \eta$. Then

$$\begin{aligned} \lim_{t \rightarrow \infty} \langle p_1^2 \rangle_A &= (k_{12}^0/\lambda^0)^2 + (k_{12}^0)^2/2\lambda^0 \tau_1 \\ &= \langle p_{1,0}^2 \rangle_A. \end{aligned} \quad (15)$$

The significance of this result is that in a system where correlation effects are absent we should have

$$\langle p_1^2 \rangle_A = \langle p_1 \rangle_A^2. \quad (16)$$

Here, we have a broadened density function; the second moment is increased by

$$(k_{12}^0)^2/(2\lambda^0 \tau_1).$$

This is the variance of the distribution.

The effect of a sinusoidal variation of temperature

Let

$$T = T_0 + B \cos ft. \quad (17)$$

T is about 300°K. in most biological reactions while B is about 10°C.; therefore, the ratio T/B is about 30. One can then write

$$A e^{-E/RT} \simeq k(T_0) \left[1 + \frac{EB \cos ft}{RT_0^2} \right], \quad (18)$$

where

$$k(T_0) = A e^{-E/RT_0}. \quad (19)$$

This time variation of the temperature does not affect $\langle p_1 \rangle_A$. However $\langle p_1^2 \rangle_A$ changes to

$$\begin{aligned} \lim_{t \rightarrow \infty} \langle p_1^2 \rangle_A &= \left(\frac{k_{12}^0}{\lambda^0} \right)^2 + \left(\frac{k_{12}^0}{2\lambda^0 \tau_1} \right) - \frac{2\lambda^0 f}{f^2 + \tau_{13}^2} \left(\frac{BA_{12}^0 G_{12}^0}{RT_0^2 \lambda^0} \right)^2 \\ &+ \frac{BA_{12}^0 G_{12}^0 \lambda^0}{RT_0^2 \tau_{13} (\lambda^{02} - f^2)} [\sin 2ft - \left(\frac{f}{\lambda^0} \right) \cos 2ft] \end{aligned} \quad (20)$$

where τ_1 is defined as before.

But

$$\tau_{13} = \tau_{11} + \tau_{12};$$

and τ_{11}, τ_{12} are defined by the correlation functions:

$$\langle A_{12}(\xi) A_{12}(\eta) \rangle = \langle A_{12}^2 \rangle e^{-\tau_{11} w} = (A_{12}^0)^2 e^{-\tau_{11} w}, \quad (21)$$

$$\langle E_{12}(\xi) e^{-E_{12}(\xi)/RT_0} E_{12}(\eta) e^{-E_{12}(\eta)/RT_0} \rangle = (G_{12}^0)^2 e^{-\tau_{12} w}. \quad (22)$$

We note the existence of a 'forced motion' term $\{\sin 2ft - (f/\lambda^0) \cos 2ft\}$ due to the periodic variation in the temperature. We can eliminate this term by taking a time average over one cycle.

$$\langle \lim_{t \rightarrow \infty} \langle p_1^2 \rangle_A \rangle_{\text{one cycle}} = \langle p_{1,0}^2 \rangle_A - \frac{cf}{\tau_{13}^2 + f^2}, \quad (23)$$

where

$$C = (2/\lambda^0) (A_{12}^0 G_{12}^0 B/RT_0^2)^2. \quad (24)$$

Equation (23) displays a reduction in the variance induced by temperature variation. We will now give a brief analysis of this important result. The distribution will be narrowest for $f = \tau_{13}$. One can show that for all reasonable values of the constants involved the second moment will have a value intermediate between the case where no correlated fluctuations exist and where $f = 0$. Note that the narrowing goes to zero as f or B go to zero. The narrowing is a function of the parameters of the system, such as τ_{11} , τ_{12} , τ_{13} , A and G . This is why this approach might be fruitful in investigating complex molecular systems.

Generalization of the results for a specialized many component system

So far we have considered an ensemble of two enzyme systems (or cells). We now generalize these results to an ensemble of N -component systems. Each N -component system is now referred to as a 'cell' and the ensemble of such cells is referred to as a colony of cells. That is, if

$$p = (p_1, p_2, \dots, p_N), \quad (25)$$

where

$$\sum_{i=1}^N p_i = 1, \quad (26)$$

$$\frac{d}{dt} \mathbf{p}(t) = \begin{pmatrix} k_{11} & \dots & k_{1N} \\ \vdots & & \vdots \\ k_{N1} & \dots & k_{NN} \end{pmatrix} \begin{pmatrix} p_1 \\ \vdots \\ p_N \end{pmatrix} \begin{pmatrix} (t) \\ (t) \\ (t) \end{pmatrix}. \quad (27)$$

Let every cell in the colony be denoted by a superscript. Then $P^a(t)$ denotes the composition of the a th cell at time t . At any time t , one can determine the average molar concentration of any species (i , say) to be

$$p_{0i}(t) = 1/M \sum_{a=1}^M p_i^a(t) \quad (M = \text{no. of cells in colony}).$$

The lack of uniformity (synchrony) in the colony can be indicated by

$$\Delta_i(t) = \langle |P_i(t) - P_{0i}(t)| \rangle_a,$$

and

$$d^2 = \langle \overline{P_i - P_{0i}}^2 \rangle_a.$$

As before, we want to show that the correlated fluctuations in the k_{ij} 's lead to an increase in the value of the second moment $d^2(t)$ and that a periodic temperature variation leads to a narrowing in the distribution, i.e. a decrease in the value of $d_i^2(t)$. The solution (Bellman, 1953) of such a system of first-order differential equation is of the type

$$\mathbf{p}(t) = \mathbf{C}\mathbf{Y}(t) e^{\mathbf{B}t},$$

where \mathbf{C} is a constant matrix given by $\mathbf{P}(0) = \mathbf{C}$, and $\mathbf{Y}(t)$ contains the time-dependent portion.

Comparing this equation with equation (8) one can observe the features necessary to contribute to the broadening of $\mathbf{p}(t)^2$ due to $\langle [Y(t)]^2 \rangle_a$ being broadened by correlated fluctuations in the same sense as k_{12} though this result is more complicated.

Similarly, temperature variation will induce a decrease in the value of $\langle [Y(t)]^2 \rangle_a$. This argument is naturally not rigorous. The more general solution of the problem will be postponed for a later publication.

Synchronization of cell colonies by periodic variation of light intensity

Zeuthen (1958) reviewed experimental observations on colony synchronization by periodic variation of light intensity. It is simple to reduce this problem to the one already discussed above. Consider the same ensemble of two-component systems except that the processes are now both catalytic and photochemical, i.e.

$$E_1 + h\nu_1 + (1) \xrightarrow{a_{11}} (2),$$

$$E_2 + h\nu_2 + (2) \xrightarrow{a_{21}} (1).$$

Then

$$dP_2/dt = a_{12}p_1I_1 - a_{21}p_2I_2,$$

$$p_1 + p_2 = 1,$$

therefore

$$dp_1/dt = a_{21}I_2 - \lambda p_1.$$

If I_1 and I_2 are constant we find

$$p_1 = p_1(0) \exp \left[- \int \lambda dt \right] + \exp \left[- \int \lambda dt \right] \int [a_{12}I_2 \exp \left[\int \lambda dt \right]] dt,$$

which is identical in form with equation (8). However, if (I_1, I_2) are time-dependent we obtain an equation similar to the one we were led to through equation (18); except for minor changes in the parameters involved in our equations (23, 24) we obtain similar results as before.

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Physiological Relationships of Rapidly Growing Mycobacteria

Adansonian Classification

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(Received 24 August 1960)

SUMMARY

The physiological properties of a collection of rapidly growing acid-fast bacilli were analysed according to a simple mathematical method proposed by Sneath. Closer relationships were observed between *Mycobacterium smegmatis* and *M. phlei* than between either of them and *M. fortuitum*; all three species form natural groups. On the basis of the Adansonian classification two other small groups were found which probably are new species. Some unnamed strains which could not be included in any of the groups mentioned above were placed in the classification and collectively labelled irregular branch. A key for the rapid identification of the specific groups is described.

INTRODUCTION

The characterization and taxonomic position of mycobacteria have been a constant problem, with numerous proposals for an adequate classification taking into account different view-points. Cultural and physiological properties, cytochemical tests, pathogenicity and antigenic relationships have been considered by many authors (Frey & Hagan, 1931; Pinner, 1932; Thompson, 1932; Gordon, 1937; Gordon & Hagan, 1938; Uesaka, 1956; Parlett & Youmans, 1956, 1958; Kushner, McMillen & Senderi, 1957; Shepard, 1957; McMillen & Kushner, 1959; Vogel, 1959; Wayne, 1959; Bönicke, 1958, 1960).

Three well-characterized species are recognized among the rapidly growing acid-fast bacilli: *Mycobacterium smegmatis* (Trevisan), Lehmann & Neumann, *M. phlei* Lehmann & Neumann and *M. fortuitum* Cruz. Gordon & Smith (1953, 1955) reclassified into these three species many others which previously had been given other names. The present study aims at an analysis of the physiological properties and relationships of a number of strains whose gross and microscopic characters make them seem similar to established species of rapidly growing mycobacteria, as well as the determination of their taxonomic position by the method proposed by Sneath (1957*a, b*) which has given good results in similar studies (Hill, 1959; Bojalil & Cerbón, to be published).

METHODS

The strains studied are listed in Table 1. They were maintained in Löwenstein-Jensen medium (Wheeler, 1951) and checked for viability and purity by microscopic examination and subcultures in different media. Single cell cultures of the strains were used to test their physiological properties:

Table 1. *Strains of Mycobacterium studied*

No. in tables	Name	Source and number
1	<i>M. smegmatis</i>	Rutgers University, S. A. Waksman
2	<i>Mycobacterium</i> sp.	UPHG, A-57-439 lymph node
3	<i>M. phlei</i> st.	Tuberkulose Forschung Institute, Börstel, Bönicke, 169
4	<i>M. ranae</i>	National Trudeau Bank, W. Steenken Jr, Trudeau Labs.
5	<i>M. lacticola</i>	Escuela Nacional de Agricultura, Chapingo, México, Etchegaray
6	<i>M. ranae</i> 110	National Trudeau Bank, W. Steenken Jr., Trudeau Labs.
7	<i>Mycobacterium</i> sp.	607, Rutgers University, S. A. Waksman
8	<i>M. friedmanii</i>	ATCC 114
9	<i>Mycobacterium</i> sp.	599 Rutgers University, S. A. Waksman
10	<i>Mycobacterium</i> sp.	ATCC 65
11	<i>Mycobacterium</i> sp.	Soil, Escuela Nacional de Agricultura, Chapingo, México Etchegaray
12	<i>Mycobacterium</i> sp.	362, ATCC, received as <i>M. butyricum</i>
13	<i>M. stercoris</i>	ATCC, 281
14	<i>Mycobacterium</i> sp.	UPHG, sputum,* C-912
15	<i>Mycobacterium</i> sp.	UPHG, sputum, C-153
16	<i>Mycobacterium</i> sp.	UPHG, peritoneum,* A-57-429-1
17	<i>Mycobacterium</i> sp.	Facultad de Medicina de El Salvador, Alfonso Trejos, subcutaneous abscess
18	<i>Mycobacterium</i> sp.	UPHG, lung cavity, A-59-52-1*
19	<i>Mycobacterium</i> sp.	UPHG, lung cavity, B-57-1787*
20	<i>M. phlei</i>	Strains NTB and W, Trudeau Labs., W. Steenken Jr. and Rutgers University, S. A. Waksman
21	<i>Mycobacterium</i> sp.	UPHG, lung, B-59-637*
22	<i>Mycobacterium</i> sp.	UPHG, bronchial aspiration, H.I. 4955
23	<i>Mycobacterium</i> sp.	UPHG, spinal fluid, H.I. 5987
24	<i>Mycobacterium</i> sp.	UPHG, sputum, C-227*
25	<i>Mycobacterium</i> sp.	UPHG, sputum 1450
26	<i>Mycobacterium</i> sp.	UPHG, R.D. 25 sputum
27	<i>M. butyricum</i>	ATCC 357
28	<i>Mycobacterium</i> sp.	UPHG, RD.47 ^A sputum
29	<i>M. fortuitum</i>	ATCC, 6841, Cruz original isolate, McMillen
30	<i>M. fortuitum</i>	Malta strain, from E. Agius. NCTC, 8573. Medical Research Council Unit, Oxford, England
31	<i>M. fortuitum</i>	<i>M. minetti</i> , strain of Penso, R. E. Gordon, R-480
32	<i>M. fortuitum</i>	Schumper—sputum, McMillen, 14
33	<i>Mycobacterium</i> sp.	UPHG, sputum,* Acapulco-343
34	<i>Mycobacterium</i> sp.	UPHG, sputum, Acapulco-103
35	<i>Mycobacterium</i> sp.	UPHG, sputum, Acapulco-102
36	<i>Mycobacterium</i> sp.	UPHG, sputum Acapulco-465
37	<i>Mycobacterium</i> sp.	Cornell University, Knaysi, received as <i>M. thamnopheous</i>
38	<i>Mycobacterium</i> sp.	Communicable Disease Centre, Chamblee Georgia, Kubica
39	<i>Mycobacterium</i> sp.	Escuela Nacional de Ciencias Biológicas, 58375
40	<i>Mycobacterium</i> sp.	Veterans Administration Hospital, 380, E. Runyon
41	<i>Mycobacterium</i> sp.	Veterans Administration Hospital, 518, E. Runyon
42	<i>Mycobacterium</i> sp.	Veterans Administration Hospital, 481, E. Runyon
43	<i>Mycobacterium</i> sp.	Cuba-29, Hepatic abscess, Cuba, Habana, Curbelo

UPHG, Unidad de Patología, Hospital General, México.

* Isolated together with tubercle bacilli.

Differential properties used: (1) *Acid* from glucose, galactose, mannose, fructose, lactose, maltose, sucrose, trehalose, melibiose, raffinose, L-arabinose, mannitol, sorbitol, dulcitol, *m*-inositol, erythritol and salicin. (2) *Utilization* of benzoate, citrate, succinate, tartrate, pyruvate and propionate. (3) *Temperatures of growth* 28°, 37°, 45°, 52°. (4) *Resistance* to 60° for 4 hr.

Gross and microscopic morphology, as well as the degree of acid-fastness, are not taken as differential criteria because of their high variability, even within one strain. The capacity for visible growth in 48–72 hr. in Löwenstein–Jensen medium, and in nutrient glycerinated agar was the basis for considering a strain as a rapid grower.

Acid formation from carbohydrates. The cultures were examined for acid production after 7 and 28 days' incubation at 37° on inorganic nitrogen agar, a modification of the medium of Ayers, Rupp & Johnson (1919) as described by Gordon & Smith (1953). Each carbohydrate was sterilized separately from the basal medium. Bromocresol purple was used as the indicator of acid production.

Utilization of organic acids. Modifications of Koser's citrate agar (Gordon & Smith, 1955) were made by combining 2 g. of the Na salts of benzoic, citric, succinic, pyruvic, or tartaric acids with saline solution buffered to pH 6.8, agar, 1.5% (w/v) and phenol red as indicator. The alkaline colour of the indicator after incubation at 37° demonstrates the utilization of an organic acid as a carbon source.

Temperatures of growth. The cultures were inoculated on Löwenstein–Jensen and in Proskauer–Beck liquid medium (Youmans, 1946) and incubated in water baths at 28°, 37°, 45° and 52° for 2–4 weeks and examined at intervals for growth.

Resistance to 60° for 4 hr. The cultures were inoculated on Löwenstein–Jensen and Proskauer–Beck media previously heated to 60° and incubated in a water bath at that temperature for 4 hr. They were then cooled under running water and incubated at 37° for 2–4 weeks and examined for growth.

The method developed for the Adansonian classification, as well as the system for quantitative notation, were as described by Sneath (1957*b*).

RESULTS

The results obtained from the different tests were tabulated: a strain \times strain ($i \times i$) table was prepared from the similarity indexes obtained, which were expressed in percentages. Another ($i \times i$) table was made by rearranging the strains in groups; the latter table is shown diagrammatically in Fig. 1. Consequently, a taxonomic tree was designed (Fig. 2), the branches of which may be considered as species. The three species previously characterized by Gordon & Smith (1953, 1955) remained as groups when subjected to Adansonian analysis. The diagram (Fig. 1) shows that *Mycobacterium smegmatis* and *M. phlei* (branch 1, groups 1 and 2) join together at a higher percentage S level than with *M. fortuitum* (branch 2, group 1). This indicates that these two species are more closely related than either is to the third. The distinctness of *M. phlei* is not very apparent in Fig. 1 because few strains were studied; but in Table 2 it is possible to appreciate that such distinctness exists. Intermediate strains joined independently to one or another of the three established species at variable S values. Also, one can observe the existence of two minor groups (branch 2, groups 2 and 3) related more to *M. fortuitum* than to *M. smegmatis* or *M. phlei*.

The branch named irregular is formed by strains that do not seem to be related to any of the other strains studied.

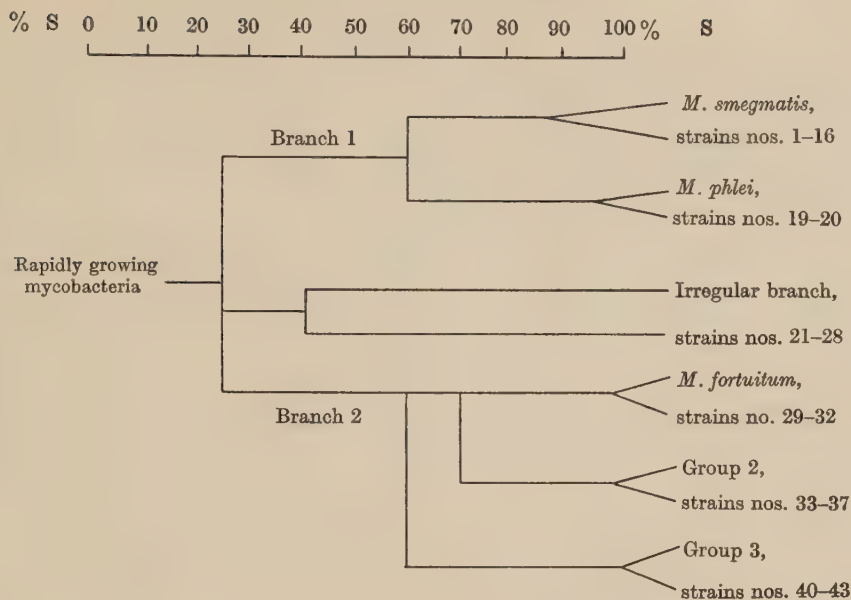


Fig. 2. Taxonomic tree of the rapidly growing mycobacteria. In another paper (Bojalil & Cerbón, to be published) the group 3 from branch 2 was named group 2b. The existence of a new group (group 2, branch 2) more closely related to *M. fortuitum* made necessary the above-mentioned change.

DISCUSSION

The three species *Mycobacterium smegmatis*, *M. phlei* and *M. fortuitum* constitute natural groups and can be accepted as logically classified. The Adansonian classification, on the other hand, clearly indicates that *M. smegmatis* and *M. phlei* are much more closely related to each other than they are to *M. fortuitum*.

Branch number 1 is formed by micro-organisms which in general are non-parasitic, capable of utilizing a great variety of carbohydrates. This group of micro-organisms was mostly isolated from soil, plants or animals, and includes *Mycobacterium smegmatis* and *M. phlei*.

Branch number 2 includes micro-organisms potentially parasitic, capable of utilizing only a limited number of carbohydrates, which were isolated mostly from pathological material or associated with these processes; *Mycobacterium fortuitum* belongs to this branch, as well as groups 2 and 3, which are definitely characterized. These groups 2 and 3 are small and include but a few unnamed strains. According to the Adansonian classification they are to be considered new species, since they show only a loose relation to pre-established rapidly growing species. On the other hand, these strains (groups 2 and 3) are grouped at high levels of similarity ($S = 100\%$). This is in contrast to many unnamed strains showing great disparity in their characters, which were included in the branch labelled irregular. Tests depending on subjective evaluation were not included. This makes the number of tests presented here seem small, but we believe that the results obtained by this Adansonian analysis are reliable.

However, it is premature to denote groups 2 and 3 as new species and a comparative study with other groups of mycobacteria is being made.

Table 2. *General properties of rapidly growing mycobacteria*

Property	Branch 1			Branch 2		
	Group 1 <i>M. smegmatis</i>	Group 2 <i>M. phlei</i>	Irregular branch	Group 1 <i>M. fortuitum</i>	Group 2	Group 3
Pigment on Löwenstein-Jensen medium	Pale-orange	Yellow	Yellow-orange	Pale-straw	Brilliant yellow	Pale-straw
Acid from:						
Glucose	+	+	±	+	+	+
Galactose	+	+	(±)	—	—	—
L-Arabinose	+	+	(±)	—	—	—
Xylose	+	+	+	—	—	—
Mannitol	+	+	+	(±)	+	—
Sorbitol	+	+	+	—	+	—
Dulcitol	+	—	(∓)	—	—	—
Mannose	+	+	+	+	+	+
Rhamnose	+	—	(∓)	—	—	—
Fructose	+	+	+	+	+	—
m-Inositol	+	—	(±)	—	—	—
Trehalose	+	+	(±)	+	+	—
Erythritol	+	—	—	—	—	—
Utilization of:						
Benzoate	+	—	(±)	—	—	—
Citrate	+	+	(±)	+	—	—
Succinate	+	+	+	+	+	+
Pyruvate	+	+	+	+	+	+
Propionate	+	+	+	+	+	+
Growth temperatures						
28°	+	+	+	+	+	+
37°	+	+	+	+	+	+
45°	+	+	(∓)	—	—	—
52°	—	+	—	—	—	—
Resistance to 60° 4 hr.	—	+	(∓)	—	—	—

+, Positive; —, negative; (±) the majority gave positive results; (∓) the majority gave negative results.

In the irregular branch, each strain could represent a specific group by itself. However, until there is particular interest in any of them they should be simply designated *Mycobacterium* sp., vigorously or poorly fermentative, thus indicating to which of the branches they are more closely related.

The following key (Fig. 3) may serve for the identification of the different groups. One frequently observes some variability in pigmentation and rate of growth in Löwenstein-Jensen medium on primary isolation; however, these properties (pigmentation and rate of growth) can be used for a preliminary identification in subcultures.

The characters listed in the key mentioned above (Fig. 3), are the most constant for each group. If a strain is aberrant in some of its properties, it may be classified incorrectly if the key alone is used; however, the same strain could be classified according to the properties listed in Table 2.

Studies of Gordon and her colleagues (1937, 1938, 1953, and 1955 principally) on acid-fast bacilli of relatively rapid growth at 28° and 37° in ordinary culture media, have allowed the separation of these micro-organisms into three main groups, especially on the basis of temperature relationships and carbohydrate utilization.

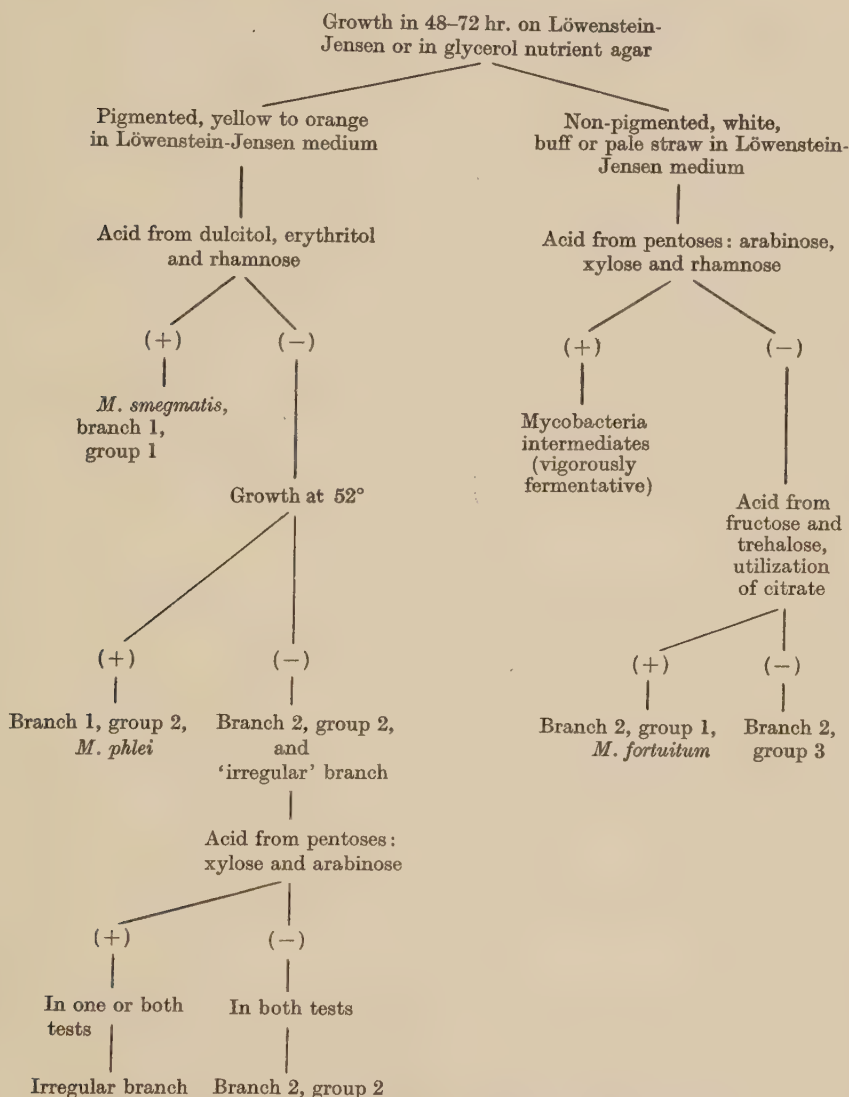


Fig. 3. Schema for the identification of rapidly growing mycobacteria.

The species *Mycobacterium smegmatis*, *M. phlei* and *M. fortuitum* are the only ones well characterized (up to the time of the latest reports). The following species, previously described as different, have been considered synonyms of one of these three species; *Mycobacterium berolinense* Bergey et al., *M. butyricum* Bergey et al., (Korn) Chester, *M. friedmanii* Holland, *M. graminis* Chester, *M. lacticola* Lehmann &

Neumann, *M. ranae* (Küster) Bergey *et al.*, *M. stercoris* Bergey *et al.*, and *M. aquae* Maië were considered as synonyms of *M. smegmatis*.

Mycobacterium giae Darzins and *M. minettii* Penso *et al.* were listed in the synonyms of *M. fortuitum*.

Groups 2 and 3 of the branch have been compared not only to those species mentioned above, but also to other species such as *Mycobacterium balnei*, *M. marinum*, *M. thamnopheous*, and *M. kansasii* and non-photochromogenic and scotochromogenic groups which will be referred to in separate reports. It should be noted, however, that group no. 3 of branch 2 differs markedly from the other studied organisms by its limited capacity to utilize sugars. This character is shown also by non-photochromogenic and photochromogenic micro-organisms. The latter, however, grow slowly, produce pigment and utilize different types of sugars (Bojalil, 1959; Bojalil y Cerbón, 1960). In particular photochromogenic *Mycobacteria* (including *M. marinum* and *M. balnei*) are capable of using fructose and occasionally galactose; but on the other hand, the non-photochromogenics do not utilize mannose.

Mycobacterium rhodochrous (Gordon, 1957) is not included in this study, it shows very weak acid-fastness and true filamentation and ramification in microcultures, features uncommon in the strains discussed in this paper, which relate it rather to the genus *Nocardia*. The amidase test (Bönicke, 1960) was negative for all strains except for those of *M. smegmatis* group for which it seems to be specific.

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Some Environmental Factors Affecting the Length of *Escherichia coli* Organisms in Continuous Cultures

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SUMMARY

The lengths of organisms of *Escherichia coli* B/r grown in continuous culture in a glucose salts medium were measured over a wide range of population densities at 22° and 37°. In some cases the cultures were exposed to continuous gamma radiation at dose rates of 600 r./hr. at 22° and of 1000 r./hr. at 37°. The average length of the forms and the distribution with respect to length depended upon the temperature, population density and radiation dose rate. The growth rate was almost independent of population density over the range in which the average length of organism showed such a marked dependence. No significant differences in growth rate were observed as between unirradiated and irradiated cultures. Changes in average length of organisms are regarded as phenotypic responses to changes in growth conditions, some conditions favouring division and others inhibiting division amongst short organisms.

INTRODUCTION

One of the advantages of a continuous culture system for bacteria is that the organisms can be maintained in active division for many generations in an unchanging environment. Such a system is very suitable for the study of the production of spontaneous or induced mutants (Novick & Szilard, 1950) and has been used in our experiments on the effects of continuous gamma irradiation on actively growing cultures of *Escherichia coli*. The effect with which we are here concerned is the appearance of long filamentous forms in the bacterial population in both irradiated and unirradiated cultures. At the outset it seemed likely that these long forms might have some properties different from those of the 'normal' shorter bacteria, and that their relative proportions would affect the behaviour of the culture as a whole. For example, the radio-resistance of the long forms might differ from that of the short ones (Lea, Haines & Coulson, 1937) or the long ones might represent the emergence of a new strain (Hughes, 1953). Very long forms have been observed in cultures subjected to gamma radiation (Lea *et al.* 1937), X radiation (Alper, 1957), ultraviolet radiation (Witkin, 1947), sudden changes in osmotic pressure (Hinshelwood & Lodge, 1944), antibiotics (Fleming, Voureka, Kramer & Hughes, 1950) and growth inhibitors (Spray & Lodge, 1943). In all these cases the additional factor superimposed on the environmental conditions was responsible for the inhibition of normal cell division. Some inhibition of division must also occur in cultures grown

in broth in the usual way since large differences in length as between individual organisms are found. The average size of bacteria in batch cultures also depends upon the nutrient concentration and phase of growth, as shown by the systematic studies of Henrici (1928). Observations of long-organism formation in batch cultures cannot therefore be interpreted in a simple manner. The provision of an environment which remains unaltered over many generation times would appear to be a basal requirement for work in this and other fields where population changes occupy more than a very small number of generation times. A continuous culture system is admirably suited to this purpose.

Some observations on the relation between growth rate and bacterial size in continuous cultures have already been reported (Schaechter, Maaløe & Kjeldgaard, 1958; Herbert, 1959). Our own observations show a close correlation between the average size of organism and the proportion of abnormally long forms, each being dependent upon the population density, the temperature and radiation dose rate.

Growth rate and length distribution in continuous cultures

When *Escherichia coli* organisms are examined under the microscope different forms can be seen which might be appropriately described as normal rods, as chains or as filaments. Such a classification has not been attempted in the present work, and as a consequence we have found the need for an inclusive term to cover these and other forms. For convenience we have adopted the word 'cell' to cover any of these various forms. We count as one 'cell' any bacterium which is recognizably separate from its neighbours in liquid suspension and moves amongst them as one entity, although in the process it may suffer changes in internal configuration by Brownian movement.

Suppose that at time t the number of cells/unit volume of a continuous culture is N and the dilution rate, i.e. the rate of flow divided by the capacity of the culture vessel, is D . If one assumes that the culture is always uniformly mixed then

$$\frac{dN}{dt} = N(\bar{\nu} - D), \quad (1)$$

where $\bar{\nu}$ is the apparent rate at which new organisms are 'born' or the number growth rate (Powell, 1956) for the whole population. If $\bar{\nu}$ is independent of N and of t , and if D can be regarded as constant during a time interval $t - t_0$, integration of (1) gives

$$\ln (N/N_0) = (\bar{\nu} - D)(t - t_0), \quad (2)$$

where N_0 is the population density at time t_0 . Thus the graph of $\ln N$ against t should have a constant slope, the value of which will give $\bar{\nu}$ when D is known. In practice when D is kept constant and $\bar{\nu} > D$ initially, $\bar{\nu}$ will eventually decrease as N becomes large, due to exhaustion of the medium and other causes, but changes in $\bar{\nu}$ may also arise from changes in the average cell size or mass. For example, when the mass growth rate $\bar{\mu}$ of the whole culture (Powell, 1956) is constant and the average mass of the cells increases at the rate ϵ the number growth rate will be $\bar{\mu} - \epsilon$.

The average diameters of *Escherichia coli* organisms under given cultural conditions are almost independent of their length (Deering, 1958; this paper, page 25), so it will be assumed in the following analysis that the mass of a cell is proportional to its length.

A continuous culture selects in favour of rapidly dividing cells from the time it is started, but several generation times may be required before the maximum and final growth rate is reached. During this early phase the distribution of generation times amongst the population changes (Powell, 1956), and consequently the distribution with respect to length usually changes also. Both distributions eventually become stable when the population density, temperature and other factors are kept constant and no further selection occurs. Such a culture will be called a steady-state culture, although the term has meaning only in relation to the property which is stable. In our case this is the length distribution, for which an infinite number of steady states seems possible, at least in theory.

The form of the length distribution in a bacterial population is determined by the time dependence of the processes of growth and division of the individual cells. Apart from studies of the distribution of generation times amongst a population (Powell, 1955, 1958) and some isolated observations of exponential growth of cells in which division had been inhibited by radiation (Lea *et al.* 1937; Deering, 1958) little is known about these processes in quantitative terms. It is possible that some information in this direction could be gained by constructing hypothetical models of the processes and comparing the length distributions predicted from them with those observed, but this will not be attempted here. Instead, we shall assume a simple exponential growth function and apply it to two special cases which represent extreme or limiting cases of the more general one.

In an unchanging environment the mass growth rate $\bar{\mu}$ of a population is constant so we shall assume, as a first approximation, that the growth rate of each cell is $\bar{\mu}$, i.e. each cell increases in length by the factor $e^{-\bar{\mu}t}$ in time t .

(1) In the first case suppose that all cells divide at a certain length $2l_1$, and that all daughter cells have an initial length l_1 . Cells of length l_1 at time zero will have lengths given by

$$l = l_1 e^{\bar{\mu} dt} \quad (3)$$

at time dt . If at time zero there are $n(l_1)dl$ cells with lengths between l_1 and $l_1 + dl$ a fraction l_1/l of this number will have lengths between l and $l + dl$ after time dt provided they have not been washed away in the interval dt . Since the fraction surviving wash-out in this interval is $e^{-D dt}$ ($= e^{-\bar{\mu} dt}$, since $D = \bar{\mu}$ in a steady-state culture) and the number of cells in any length range does not change with time, the number between l and $l + dl$ will be

$$n(l) = n(l_1)(l_1/l)e^{-\bar{\mu} dt} dl,$$

which by (3) can be written

$$n(l) = n(l_1)(l_1/l)^2 dl. \quad (4)$$

When $n(l)$ is plotted against l/l_1 using double logarithmic scales as in Fig. 7*a* the graph has a slope -2 for $2 \geq l/l_1 \geq 1$. Outside this length range $n(l)$ is zero. In any real culture all cells would not divide at precisely the same length nor would they divide to give two exactly equal daughters. The ends of the distribution would therefore not be sharp but rounded as in the experimental curve to which Fig. 7*a* has been fitted.

(2) In the second case suppose that cells do not divide at length $2l_1$, but continue to grow longer at the same rate $\bar{\mu}$ until they reach a length ql_1 , where q is a large

positive integer. If they then divided into separate cells of length l_1 , a steady-state culture would be possible and the length distribution for $q \geq l/l_1 \geq 1$ would have a slope of -2 . This distribution is represented graphically by the line (b) of Fig. 7 for which q is assumed to be very large.

In passing, it may be noted that if divisions did not occur at one particular length but took place at all lengths greater than $2l_1$, with similar frequencies, the average cell length and the slope of the length distribution would have values between those for the two extreme cases (1) and (2). Thus the average length l_a at which cells pass through their first division would be correlated with the average length of the whole population and with the slope of their length distribution.

So far we have considered only steady-state cultures of cells behaving according to our simplified model. In practice it may not be possible to maintain the dilution rate at its steady-state value $D = \bar{\mu} = \bar{v}$ and it is therefore important to know whether short-term fluctuations in dilution rate are likely to influence the length distribution substantially. Let us suppose that a culture has been operated in a steady state with a dilution rate D_1 and that the dilution rate is suddenly increased to D_2 . If l_a is independent of dilution rate, all cells will have the same decreased chance of surviving wash-out after time t , viz. $e^{(D_1 - D_2)t}$. The change in dilution rate *per se* would therefore leave the length distribution unchanged. However, if the product $(D_1 - D_2)t$ were sufficiently large, the cell density would change appreciably and a dependence of l_a upon cell density would result in an altered length distribution.

METHODS

In the experiments described below *Escherichia coli* B/r derived from a stock kindly supplied by Miss T. Alper was grown in a glucose salts medium (Lederberg, 1950) at pH 7.0 in a continuous culture system. Starter cultures were routinely grown overnight at 37° in minimal medium and samples of such cultures were used as inocula for continuous cultures. The culture vessels had capacities of approximately 9 ml. and each was supplied with air at the rate of 700 ml./hr.⁻¹ and with nutrient at a rate which could be adjusted by manual control of the hydrostatic head h (Fig. 1). The positions of the reservoir R and mixer M having been fixed the dilution rate fell by 1–3% over 24 hr., depending upon the actual dilution rate, the area of free liquid surface in the reservoir and the head h . Normally the system was adjusted to give unrestricted growth at constant or nearly constant population density. The apparatus in the form here described therefore functioned as a crude turbidostat (Bryson, 1959). A stirrer consisting of a permanent magnet encased in stainless steel driven at 300 rev./min. provided efficient mixing of medium and air. With its free-running tyre at one end and a fixed tyre at the other, the stirrer was subject to a frictional turning couple set up by its forced revolution and this caused it to rotate about its axis. This form of stirrer always remained free from adherent bacterial growth even after running continuously for as long as 2 weeks. Gamma radiation was provided by a ⁶⁰Co source of 8 curies kindly placed at our disposal by Dr G. J. Neary.

During an experiment samples were withdrawn from the culture vessels from time to time by means of Pasteur pipettes. These samples (vol. 10⁻² ml.) were examined microscopically, with dark ground illumination and a Thoma Hawksley counting

chamber $25\ \mu$ deep. It was customary to record the total count (about 200) and also the numbers of 'long cells', i.e. cells longer than $8\ \mu$, seen over the whole or part of the ruled area. When the complete length distribution of a sample was required the lengths of all cells (about 200) within a given area were measured.

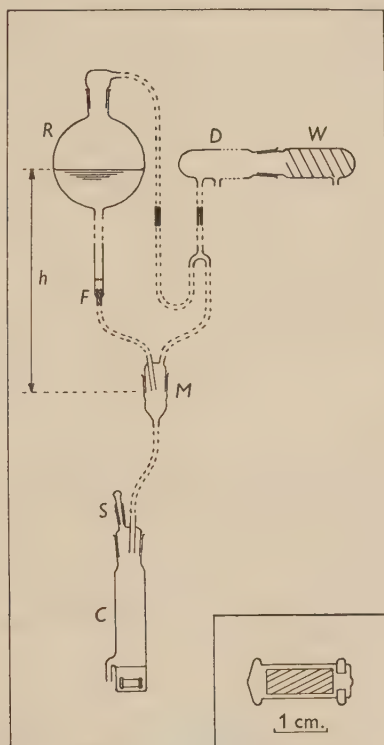


Fig. 1. Schematic diagram of the apparatus. Silicone rubber connexions between the glass components are shown by dashed lines. Heavy black lines indicate narrow tubes with appreciable flow resistance. *C* = culture vessel; *S* = sampling port; *M* = mixer; *D* = distributor of sterile humid air; *W* = cotton wool plug; *F* = filter; *R* = reservoir of medium. Inset, magnetic stirrer.

RESULTS

The results to be described relate to experiments in which two or three cultures were inoculated from the same starter culture and run simultaneously, usually at different population densities. The dilution rate of each culture was adjusted so that it was about equal to the growth rate. During the first few hours changes were observed in % long cells in the population and in the average cell length, but after several generation times both quantities reached apparently stable values. Figure 2 shows the changes in % long cells in typical cultures during this initial period. After the % long cells had become constant observations were continued during several generation times (usually at least 10) so that sufficient data relating to the length distribution of cells in the chosen steady state could be obtained. In the following paragraphs the results given are for cultures which had reached, or appeared to have reached, stable length distributions. However it must be emphasized that the

experiments were rarely continued for more than 24 hr. and therefore we have no evidence that the same effects would be found with much older cultures. The experimental errors in % long-cell values were also relatively large so that over the short periods covered by the observations it was not possible to determine whether there was a tendency for the % long cells to increase or decrease slowly.

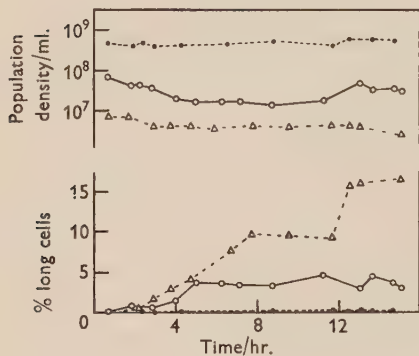


Fig. 2

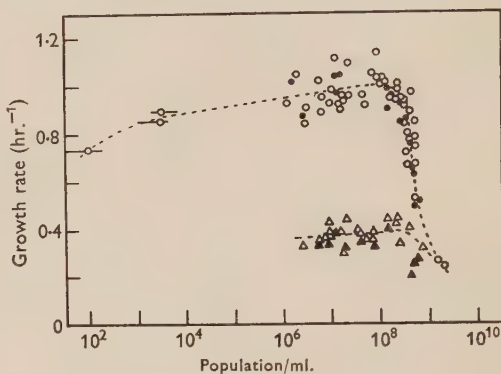


Fig. 3

Fig. 2. Changes in the proportion of cells which are long, i.e. greater than 8μ during the first few hours in three continuous cultures inoculated simultaneously from the same starter culture and run at 37° at different population densities, namely: (●) 4×10^8 /ml.; (○) 3×10^7 /ml.; (△) 4×10^6 /ml.

Fig. 3. The growth rates of cultures as functions of population density. The extent of the large variation in population density for the three most dilute cultures is indicated by horizontal lines. Unirradiated cultures at 22° (△) and 37° (○); irradiated cultures at 600 r./hr. at 22° , (▲) and at 1000 r./hr. at 37° (●).

Growth rates

The number growth rate, which becomes equal to the mass growth rate in a steady-state culture, was calculated from experimental data by using equation (2). All values of growth rate for cultures grown at 22 and 37° , with and without gamma irradiation, are plotted in Fig. 3. It was estimated that they were subject to errors of about 5%, due mainly to temperature fluctuations and to errors in the measurements of culture vessel volumes. Although there were wide differences between the results from experiment to experiment, a definite progressive decrease in growth rate at 37° was evident at population densities greater than 2×10^8 /ml. due presumably to partial anoxia or nutrient limitation. A similar trend appeared at 22° at a rather higher population density. At 37° observations were extended to population densities of about 10^4 to 10^2 cells/ml. by making viable counts (colonies at 72 hr. at 37° on salts glucose agar) instead of the usual total counts.

Although growth rates for irradiated cultures were somewhat smaller on the average than those for unirradiated cultures at the same population density, the differences are not statistically significant.

Proportion of long cells amongst population

Figure 4 shows that there was an almost unique relation between the average cell length and % long cells, particularly for unirradiated cultures. Since both of these

quantities are dependent upon the length distribution, it follows that either could be used to specify such a distribution.

At 37° the % long cells decreased towards zero as the cell concentration (population density) increased in the observed range, namely 10^6 to 10^9 /ml. This trend could be readily demonstrated in a single experiment such as the one represented in Fig. 2 in which three cultures from the same inoculum were run simultaneously at different population densities. However, when the results from many experiments were pooled in one diagram (as in Fig. 5), it became clear that there was not a unique relation between % long cells and population density.

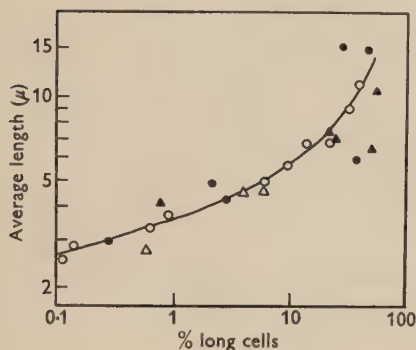


Fig. 4

Fig. 4. The average length of cells in a culture as a function of the % long cells. Points for irradiated and unirradiated cultures show no systematic differences. Symbols as Fig. 3.

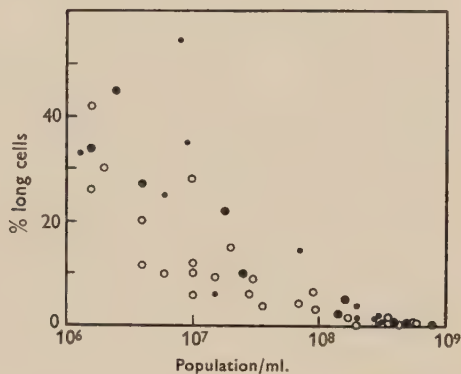


Fig. 5

Fig. 5. Observations from a large number of cultures showing the general form of dependence of % long cells upon population density at 37°. Points corresponding to cultures grown under continuous gamma irradiation at 1000 r./hr. (●), and 600 r./hr. (◐) tend to lie above those for cultures grown in the absence of radiation (○), but the differences are not statistically significant.

The results of Figs. 3 and 5 taken together show that the greatest changes in % long cells occurred in the range of population densities below 2×10^8 /ml. where the growth rate was almost constant. The average cell length in this population density range was therefore independent of the growth rate.

The effect of gamma radiation at dose rates up to 1000 r./hr. at 37° was tested by irradiating cultures after they had each reached an approximately steady state. The observed changes in the proportion of long cells were relatively small, and taken together did not give unequivocal evidence of an increase due to radiation at these dosages. The actual results are plotted in Fig. 5. Experiments were attempted at 43°, which is above the optimum temperature for growth, but reproducible results were not obtained.

The values of % long cells in unirradiated cultures at 22° were only about one-tenth of those values at 37° with the same population density. For this reason and also because fewer observations were made, the evidence for a dependence of % long cells on population density at 22°, similar to that of Fig. 3, was not conclusive. However, the presence of such a small proportion of long cells in an unirradiated

culture made the detection of a small increase due to gamma radiation correspondingly easier. The results of an experiment with cultures at two different population densities exposed to gamma radiation (600 r./hr.) are shown in Fig. 6. The increase in % long cells following irradiation was more marked at the smaller population density, the % long cells reaching a value typical of those for unirradiated cultures at 37°. Thus an increase in temperature and exposure to gamma rays produced similar changes in the proportion of long cells.

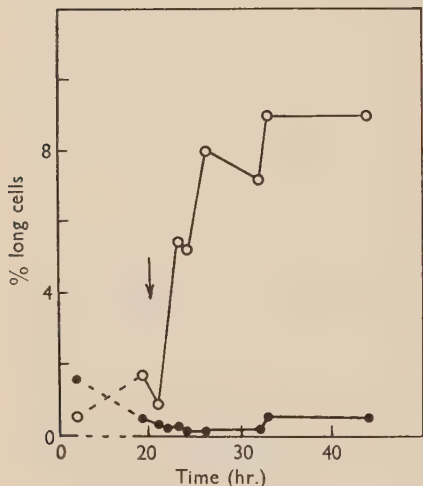


Fig. 6

Fig. 6. The effect of gamma irradiation at 600 r./hr. on % long cells in cultures at 22°, at high (3×10^8 /ml., ●) and low (3×10^7 /ml., ○) population densities. Irradiation commenced at 20.6 hr.

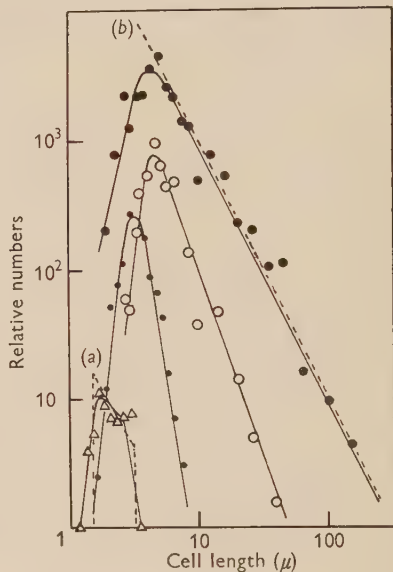


Fig. 7

Fig. 7. The number of cells observed/unit interval of length plotted against length for each of four cultures at 37°. The population densities were 10^9 /ml. (Δ), 4×10^8 /ml. (●), 1.0×10^7 /ml. (○) in the absence of gamma radiation, and 1.2×10^7 /ml. (●) at 1000 r./hr. gamma radiation. The points at longer cell lengths represent average values of the frequency over several adjacent unit length intervals. The graph (a) showing the distribution to be expected on the simplest assumptions (see text) has been fitted to one set of experimental points. The line (b) shows the expected distribution for a population in which cells only divide when they are very long.

The general form of the length distribution of cells of *Escherichia coli* strain B/r in continuous cultures is shown in Fig. 7. The number of cells with lengths between l and $l+dl$ divided by dl , which may be denoted by $n(l)$, is plotted against l , using double logarithmic scales. At lengths greater than the mode, the distribution curves became linear within the limits of experimental error. An analysis of all the data showed that the slope of the linear portion and the average cell length were correlated (coefficient 0.76, $P < 0.001$) despite the wide range of conditions under which the cells were cultivated. It follows that the shapes of all distribution curves could be expressed in terms of the average length only, a conclusion independently

confirmed by the almost unique relation existing between the average length and % long cells (Fig. 4). The uppermost curve of Fig. 7 for a culture at 37° and irradiated at 1000 r./hr. is noteworthy in that the slope of its linear portion approximates to -2.0. This, on our simple model, is the smallest possible value of the average slope for a culture in a steady state and obtains when cells which have reached a certain length grow longer without further division.

Stability of cultures

Since a change in population density generally caused a change in length distribution, a culture could only become strictly stable when the dilution rate was maintained exactly equal to the growth rate. In these experiments this condition was only fulfilled at population densities greater than 2×10^8 /ml. when the system behaved as a chemostat (Bryson, 1959) with the suboptimal growth rate (compare Fig. 2) adjusting itself to equality with the dilution rate. At population densities less than 2×10^8 /ml. any error in the adjustment of the dilution rate caused a steady increase or decrease in population density (see Equation 2). Over the period of a few hours required for the establishment of a stable value of % long cells, changes in population density were relatively small, amounting to a factor of 2 or less, and the corresponding changes in % long cells to be expected on the basis of the trend in Fig. 5 were comparable to their experimental errors. The control of the population density can therefore be regarded as adequate for these experiments.

Although on some occasions the dilution rate was deliberately altered from its steady-state value by as much as 50 % for periods of one or two generation times, changes in the population of long cells were always small and could be attributed to changes in population density. Possible complications in the interpretation of length distribution as due to sudden changes in dilution rate (as discussed earlier) have therefore been neglected.

Cell diameters

The diameters of living cells were estimated visually to the nearest 0.1 μ using a $\times 90$ objective with phase contrast bright field illumination and with dark ground illumination. For 113 cells from one culture in the length range 2.5–30 μ the average diameters were $0.93 \pm 0.08 \mu$ with dark ground and $0.78 \pm 0.06 \mu$ with bright field. The difference between the two values can be ascribed to diffraction. When the measured diameters were divided into two equal groups about the median, the average diameters of the groups did not differ significantly.

Search for evidence of selection

The observations described about the dependence of average length upon temperature, population density and radiation could be explained in terms of phenotypic variation or of selection amongst clones of different average length. Experiments designed to distinguish between these two hypotheses showed that changes in the proportion of long cells due to changes in population density were reversible, whilst the proportions of long cells in cultures derived from single cells and from stock slopes did not differ significantly. An attempt to isolate a long-cell variant from a dilute culture at 37° was not successful, the progeny of single cells after 30 generation times showing no significant differences in average length despite the widely differing lengths of the parent cells.

DISCUSSION

Since we dealt exclusively with growing cultures it was not possible to determine whether the observed changes in length were due to selection or adaptation. Our failure to find evidence in our stock cultures for selection amongst two or more strains with very different tendencies to grow long forms has lead us to regard the observed changes as phenotypic responses to changing growth conditions. On this basis a change in average cell length of a population must be interpreted in terms of some change which tends to make all cells become longer or shorter. A change in average length implies a change in the length at which cells on the average divide; thus our experimental observations are broadly consistent with the simple model proposed above, if the average length at which cells divide is conditioned by the temperature, population density and the degree of gamma irradiation.

At 37° a change in population density from 10⁶/ml. to 2 × 10⁸/ml. produced no significant change in growth rate but a marked decrease in average cell length. A similar change in population density at 22° also resulted in a decrease in cell length, but again the growth rate remained constant. Thus, a dependence of average cell size upon growth rate which Schaechter *et al.* (1958) found with *Salmonella typhimurium* certainly does not hold for *Escherichia coli* in minimal medium at population densities less than 2 × 10⁸/ml. On the other hand, when the results of Schaechter *et al.* (1958) for *S. typhimurium* are combined with similar ones for the dry weight of *Aerobacter aerogenes* and *Bacillus megaterium* (Herbert, 1959) and our own for *E. coli*, one finds that in all cases the forms of the dependence of size upon growth rate are almost identical in the region of suboptimal growth rates. We interpret this as indicating that the relation found by Schaechter *et al.* (1958) applies only when there is limitation of growth rate by partial exhaustion of the medium or limitation by accumulation of toxic substances.

The dependence of the % long cells upon population density found in the present experiments suggests that the bacteria alter their growth medium in such a way as to inhibit the formation of long cells. This dependence is consistent with the hypothesis that bacteria remove an inhibitor present in the minimal medium or with the alternative hypothesis that they excrete a metabolite into it. Since gamma radiation also affects the % long cells, particularly at 22°, the hypothetical inhibitor or metabolite may be a compound associated with a radiosensitive metabolic sequence.

We wish to thank Mr R. J. C. Hudson for the detailed design and construction of much of the apparatus.

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The Nature and Radiation Sensitivity of the Long Forms of *Escherichia coli* Strain B/r

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SUMMARY

By suitable adjustment of growth conditions in continuous cultures, organisms of *Escherichia coli* B/r with average lengths covering a wide range were produced and studied. From X-ray survival data it was concluded that radiosensitive sites were distributed along the length of an organism at intervals of 1–1.5 μ , whilst nuclear staining by the HCl-Giemsa method showed chromatinic bodies at an average spacing of 1.2 μ . Thus each nuclear body appeared to be a radiosensitive site. Lysozyme treatment did not reveal evidence of transverse membranes apart from those at visible ‘waists’. It was concluded that the longer organisms arose by the inhibition of the terminal stages of division. This inhibition did not affect the multiplication of the nuclear and cytoplasmic components, although there was considerable aggregation of nuclear material in irradiated organisms.

INTRODUCTION

When *Escherichia coli* strain B/r is cultivated in a glucose salts medium the average length of the organism (‘cells’ in sequel; see Maclean & Munson, 1961) can be altered by changing the temperature and the population density and also by gamma irradiation (Maclean & Munson, 1961). By adjustment of these environmental factors one could therefore readily compare the properties of *E. coli* strain B/r rods of different lengths, and investigate any specific effects associated with growth under gamma irradiation. From studies of X-ray survival and of the number and disposition of nuclear bodies and cell membranes, some progress has been made towards an understanding of the nature of the long forms and the effect of radiation on their division processes.

METHODS

The present experiments were carried out with *Escherichia coli* strain B/r cultivated in a continuous system in a glucose salts medium as described earlier (Maclean & Munson, 1961). Cultures were grown at 22° and 37° and in some experiments they were irradiated continuously with ⁶⁰Co gamma radiation at dose rates of 600 r./hr. at 22°, and 1000 r./hr. at 37°. Adjustment of the bacterial concentration (population density) provided a simple means of controlling the average length when the temperature and radiation dose-rate had been fixed. In each experiment growth was continued for at least 10 generation times under the selected conditions in order to allow the length distribution amongst the population to become stable. Samples were then drawn from the culture vessel with Pasteur

pipettes, one to be X-irradiated and the other for length measurements of 100 to 300 living cells. In some cases a third sample was taken for nuclear staining.

Survival after X-irradiation. The sample for X-irradiation was diluted to a population density of 10^5 /ml. in ice-cold sterile minimal medium (Lederberg, 1950) prepared some hours previously. Samples of the suspension were put into small glass tubes cooled in ice and water, and exposed to X-ray doses of 0, 2, 5, 10 and 20 kr. delivered at a dose rate of 550 r./min. The radiation half value layer was 1.2 mm. copper. After irradiation 0.1 ml. samples were spread on minimal medium containing 2% (w/v) agar. The plating was carried out at room temperature as rapidly as possible and incubation at 37° was usually started within 30 min. of the end of the irradiation. Final counts of colonies were made after incubation for 72 hr. Amongst cultures which were not exposed to X-radiation the population of viable cells was not significantly less than unity (0.95 ± 0.21).

Although our procedure of plating at room temperature was convenient it was open to the objection that recovery or restoration during the interval before the cells reached 37° might have altered the proportion of cells surviving and hence also the shapes of survival curves (Stapleton, Billen & Hollaender, 1953; Alper & Gillies, 1960). The ratio (colony count):(colony count at zero restoration time) which may be termed the restoration factor, was therefore measured at room temperature for restoration times up to 4 hr. The restoration factor increased progressively with dose of radiation and with restoration time up to about one generation time. There was, however, no evidence of a change in shape of survival curves, the smaller alterations in the surviving fractions after 30 min. restoration being equivalent merely to an expansion of the dose scale by a few %. We conclude that any errors in the estimation of surviving fractions due to partial restoration were of the same order as the overall errors due to other causes. No correction for restoration was therefore made.

Nuclear staining. Staining was carried out by a variation of the HCl-Giemsa method (Robinow, 1944). Smears were fixed in osmic acid vapour (3 min.), placed in Schaudinn's ethanol solution ($1\frac{1}{2}$ min.) and stored overnight in iodine + ethanol. The following day the slide was placed in N-HCl at 55°–60° (10 min.), washed with M/400 phosphate buffer (pH 7.0), stained with Giemsa (37° for 4 hr.) and dried and mounted in Canada balsam.

Digestion of bacterial DNA with DNA-ase. Two bacterial smears were made on one slide and these were dried and fixed in acetic acid + ethanol (1 + 3). On the following day a depression slide containing a solution of DNA-ase (DNA-ase, once recrystallized; Nutritional Biochemical Corpn.; 150 μ g./ml.; MgSO_4 , 1.75×10^{-3} M in 0.06 M-phosphate buffer, pH 7.2) was mounted over one smear and over the other smear a similar depression slide without DNA-ase. The slides were sealed with paraffin and incubated at 37° for 30 min. The depression slides were then removed and staining continued as above. The DNA-ase activity was tested by the method of McDonald (1955). No RNA-ase was detected by a modification (Dr J. E. Stanier, personal communication) of the method of Bernheimer & Steele (1955).

Preparation of osmotically sensitive spheres

Cell suspensions were filtered through a membrane filter (Oxoid) and washed with 10^{-3} M-phosphate buffer (pH 7.0) and then with 0.1 M-2-amino-2-hydroxymethyl-

propane-1:3 diol (tris) buffer (pH 8.0). A few drops of a solution of lysozyme (100 μ g. lysozyme/ml., Nutritional Biochemical Corp., Cleveland, Ohio, U.S.A.; 200 μ g. ethylenediaminetetra-acetic acid/ml., in 3×10^{-2} M-tris buffer (pH 8.0) and 0.5M sucrose; Mahler & Fraser, 1956) was applied to the paper and the cells brought into suspension by stirring with a platinum loop. A few loopfuls were then used to fill a depression slide (100 μ deep), which was sealed with a coverslip and cedar wood oil. The progress of the action of the lysozyme was then followed microscopically and photographs taken of the spheroplasts which had settled to the bottom of the depression.

RESULTS

Shapes of X-ray survival curves

The proportion of cells able to form colonies on minimal agar after X-irradiation was found to be a function of the dose of X-radiation and the average cell length; some typical results are shown in Fig. 1, where the logarithm of the surviving fraction is plotted against the dose of X-radiation. For the shortest cells the survival curve is almost exponential but as the average length increases the shoulders become more and more marked. By drawing smooth curves through the experimental points instead of the broken lines as in Fig. 1, the doses of X-radiation for any degree of survival (or killing) can be found. The shapes of the survival curves can then be conveniently specified in terms of the ratio of the X-irradiation doses at two arbitrarily chosen degrees of killing. It appears from Fig. 2 that the ratio of the doses for 90 and 50% killing, namely, LD90/LD50, depended mainly upon the average cell length and was independent of the cultural conditions within the rather wide limits set by the scatter of the points.

The graphs of Fig. 1 are similar in form to the family of survival curves which can be derived theoretically on certain simple assumptions which are formally equivalent to those of Lea (1946) and Atwood & Norman (1949). These assumptions are: (a) each cell consists of an integral number, r , of units which can be independently inactivated by radiation; (b) the probability of inactivation of a unit is $(1 - e^{-\lambda D})$ where λ is its radiosensitivity and D is the radiation dose; (c) a cell remains viable in the sense that it can give rise to a colony provided that one or more of its units are not inactivated.

Whatever the nature of the units, it can readily be shown that the fraction S_r of cells with r units/cell which survive a dose D is given by

$$S_r = 1 - (1 - e^{-\lambda D})^r, \quad (1)$$

whence it follows that

$$\frac{\text{LD90}}{\text{LD50}} = \frac{\ln(1 - (\frac{9}{10})^{1/r})}{\ln(1 - (\frac{5}{10})^{1/r})}. \quad (2)$$

Comparison of the calculated values of the ratio LD90/LD50 for different values of r , shown on the scale to the right of Fig. 2 with the observed ratio LD90/LD50 for cells of different average length, indicates that r is approximately proportional to the average cell length. Thus $r = 8$ corresponds to a length of 12 μ , $r = 4$ to 6.5 μ , $r = 2$ to 3.5 μ , and $r = 1$ to approximately 2 μ . The survival data are therefore consistent with our assumptions if the units of which a cell is composed each occupy on the average about 1.5 μ of its length. Since it has been assumed that a colony

can arise from only one unit, each unit must contain one nucleus at least. It seems therefore likely that stained nuclei may prove to be useful markers of the assumed functionally separate cellular units.

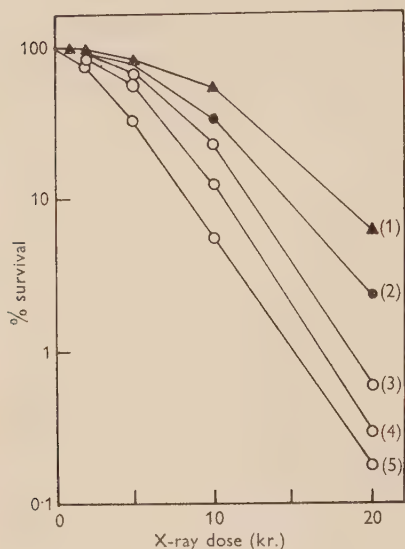


Fig. 1

Fig. 1. X-ray survival curves of *Escherichia coli* strain B/r of different average lengths. The average cell lengths and culture conditions were: curve (1), $\bar{l} = 11.0 \mu$; 22° , 600 r./hr.; curve (2), $\bar{l} = 16.1 \mu$; 37° , 1000 r./hr.; curve (3), $\bar{l} = 11.8 \mu$; 37° , no gamma radiation; curve (4), $\bar{l} = 7.3 \mu$; 37° , no gamma radiation; curve (5), $\bar{l} = 3.1 \mu$; 37° , no gamma radiation.

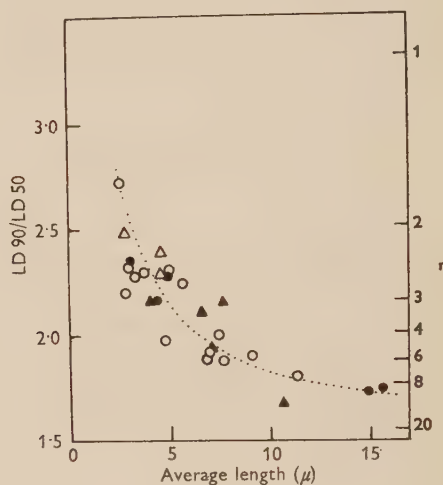


Fig. 2

Fig. 2. The shape of X-ray survival curve of *Escherichia coli* strain B/r as a function of the average length. The shape is expressed in terms of the ratio of the doses for 90 % killing and 50 % killing. \circ = Cultures at 37° with no radiation; \bullet = cultures at 37° with gamma radiation (1000 r./hr.); \triangle = cultures at 22° with no radiation; \blacktriangle = cultures at 22° with gamma radiation (600 r./hr.).

Nuclear staining and DNA-ase treatment

Plate 1, fig. 1, shows cells from a culture at 22° at a population density 7×10^8 /ml. grown without gamma irradiation. The cells are very short and have only one or two nuclei. At 37° and a population density of 10^7 /ml. (Pl. 1, fig. 2) the cells are generally much longer and contain several nuclei, mostly grouped in pairs. Under gamma irradiation cells grown at a similar population density at 22° (Pl. 1, fig. 3) and 37° (Pl. 1, fig. 4) have a completely altered appearance. The regular pattern has largely disappeared and the nuclear material occurs in large masses, often spaced irregularly. In some cases all the nuclear material is gathered into one or two areas and in others there appear to be no nuclei. Many of the shorter cells appear almost unaffected by the radiation.

Photographs of more than 1000 cells at a magnification of $\times 7070$ were examined and the number of deeply-stained spots of diameter about 0.4μ (which were assumed to be nuclei) and the cell length type recorded. In many cases spots of this size did not appear clearly separated from each other, and the number of nuclei scored was

then decided by subjective criteria such as the number of condensations within the deeply stained areas or by the lengths of the stained areas when no condensations were visible. The average lengths of cells containing 1, 2, 3, ..., nuclei grown under a variety of environmental conditions are shown in Fig. 3. Points for cultures grown at 22° and 37°, with and without gamma radiation, all lay close to one straight line through the origin. When one selects from the 1150 photographs those in which all nuclei are clearly visible (383) the points obtained also fit the same line.

Although gamma irradiation during growth had a characteristic effect on the appearance of stained preparations some difficulty was encountered in finding simple criteria by which the changed appearance could be expressed quantitatively. To minimize subjective errors it was decided that areas of staining should be scored rather than numbers of individual nuclei. At 37° significant differences between irradiated and unirradiated cultures were found for: (a) the proportion of cells having no stained areas (Table 1; $P < 0.01$); (b) the proportion of cells having one central stained area only (Table 1; $P < 0.01$); (c) the median lengths of cells having between 1 and 6 areas/cell ($P < 0.01$). A comparison of preparations of cells treated with and without DNA-ase showed that the enzyme treatment removed most of the stain from the deeply stained areas which had been regarded as nuclei. There seems therefore no reasonable doubt that these were the regions which contained most of the DNA.

Table 1. *Proportions and average lengths of Escherichia coli strain B/r in cultures grown with or without gamma irradiation which show: (1) no nuclear stained areas; (2) one such central area only*

Continuous cultures at 37° and population densities between 10^8 and 2×10^8 /ml. Dose rates zero or 1000 r./hr. Lengths are in mm. on prints at magnification $\times 7070$.

Gamma radiation	Total no. cells observed	Cells with only one central stained area		Cells with no stained areas	
		No.	Av. length (μ)	No.	Av. length (μ)
—	548	6	17	3	14
+	457	40	22	25	18

Shrinkage in bacterial preparations and the average cell length/nucleus

It was found that the fixing, staining and mounting of the bacteria caused them to shrink in length and breadth. To find the average spacing of nuclei in a living cell it was therefore necessary to estimate the shrinkage factor. This was done in two ways: (a) by comparing the median values for the distributions of living and stained cells with respect to overall length; (b) by comparing the average lengths of segments into which cells were divided by visible 'waisting' in the living preparation and by incomplete cross-walls in the stained preparations. Consideration of the likely errors leads to the expectation that (a) would yield a falsely high value and (b) a falsely low value. The average values for the factor were: (a) 1.67 ± 0.16 ; (b) 1.26 ± 0.08 . We have adopted the factor 1.5 as the best estimate.

The average distance between nuclei in the photographs, as given by the straight line of Fig. 3, is 0.57 mm.; allowing for shrinkage and magnification the actual distance is therefore $(1.5 \times 0.57)/7070$ mm., i.e. 1.21μ . This is sufficiently near

the rough figure of 1.5μ for the average length occupied by one radiosensitive unit as found from the data of Fig. 3 to suggest that the numbers of nuclei and radio-sensitive units are equal.

Observations on cells treated with lysozyme

Under the action of lysozyme the cell wall loses its rigidity (Sistrom, 1958) and the remains of cytoplasm within the cell membrane tends to become spherical (= spheroplast) in media of suitable osmotic pressure. Visual observations of *Escherichia coli* strain B/r showed that a high proportion of the shorter cells became spherical after 20–40 min. treatment with the lysozyme solution described (Methods) at room temperature. The average diameter of the spheres appeared to be insensitive to changes in sucrose concentration over the range 0.2 – $0.5M$, but the observations were not sufficiently precise to rule out a slight dependence of size upon sucrose concentration. Cells initially composed of two segments changed to pairs of spheres which often remained in close contact, showing that although the membrane had divided, cell division was not complete. In a γ -irradiated culture a very long cell which was not segmented was seen to swell simultaneously at two points. One sphere grew more rapidly than the other and burst, whereupon the contents of the rest of the cell were rapidly lost through the open end. This observation suggested

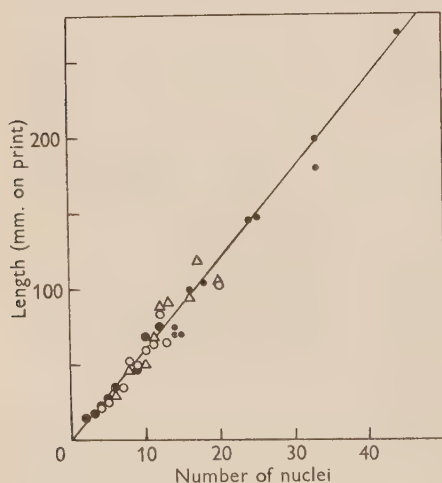


Fig. 3

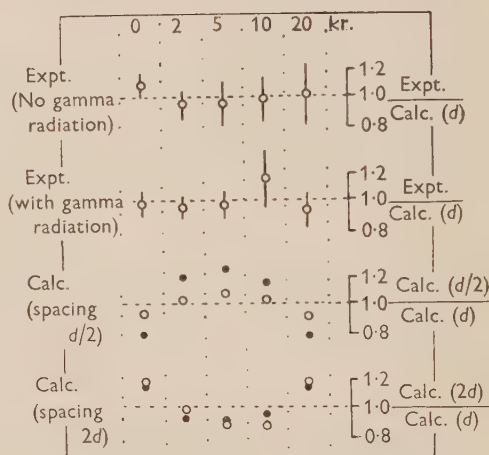


Fig. 4

Fig. 3. The relation between cell length and the number of nuclei for *Escherichia coli* strain B/r. Data from photographs. Observations for cells with a particular number of nuclei are pooled and the average length plotted. From all cells of unirradiated cultures, \circ ; from all cells of γ -irradiated cultures, \triangle ; from all cells of all cultures with less than 3 observations/point, \bullet ; from those cells of all cultures in which all nuclei are separately visible, \bullet .

Fig. 4. Test for systematic deviations of experimental points from calculated survival curves of best fit. At each X-ray dose deviations found in individual experiments were pooled and the mean deviation with its standard error plotted in the upper half of the figure. Data for γ -irradiated and unirradiated cultures are shown separately. Calculated curves were derived from the corresponding length distributions assuming that each radio-sensitive unit occupies a cell length d ($= 1.2 \mu$). The effect of a change in the assumed nuclear spacing by a factor of 2 either way is shown in the lower half of the figure for typical populations of short (\bullet) and long (\circ) average length.

that the absence of constrictions of the cell wall was probably associated with absence of transverse septa.

Measurements on a large number of cells from one culture showed that the distributions with respect to volume before and after exposure to lysozyme were very similar except at large volumes, where the discrepancy was consistent with a relatively high probability of bursting. The average cell length, the average segment length and the average length of a rod of the same diameter as a normal cell and equal in volume to the average type, are recorded in Table 2 for three continuous cultures grown from the same parent culture and operated simultaneously. The close agreement between the figures in the last two columns implies that when a segment becomes spherical the change in volume must be relatively small.

Table 2. *Sizes of whole cells, segments and the osmotically-sensitive spheroplasts derived from them by lysozyme treatment*

For comparison purposes the average volume of the spheroplasts is expressed in terms of the length of a rod-shaped cell having the same volume.

Cultural conditions			Average cell length (μ)	Average segment length (μ)	Equivalent average length for spheroplasts (μ)
Temp.	Population density	Gamma radiation			
23°	4–10 ⁸ /ml.	—	2.7	2.0	2.2
23°	10 ⁷ /ml.	600 r./hr.	9.5	7.6	6.4
37°	10 ⁷ /ml.	—	5.7	3.7	4.1

Test of the hypothesis that the number of radiosensitive units/cell is equal to the number of nuclei

Although the evidence for the equality of the numbers of nuclei and radiosensitive units presented above is sufficient to justify it as a working hypothesis, a more searching test is desirable, for the following reasons. The data of Fig. 2 are concerned with survival in the range 10–100%, which constitutes only part of the available experimental information. Also the relation between the ratio LD90/LD50 and the number of radiosensitive units/cell shown in Fig. 2 applies strictly only to a uniform population, whereas our cultures were markedly not uniform with respect to cell length (Macleane & Munson, 1961) and therefore not uniform with respect to the number of units/cell. A direct estimate of the average number of radiosensitive units/cell from the survival data could not be made by the method of Atwood & Norman (1949) because the numbers of nuclei/cell had distributions which deviated widely from a Poisson distribution. We have therefore tested our hypotheses by comparing the shape of the experimental survival curve for each culture with the corresponding shape of the calculated curve and looking for evidence of systematic discrepancies between them.

To calculate the survival curve for each culture it was first necessary to determine the distribution of the number of nuclei/cell. For each cell of length l the number of nuclei was taken as the integer nearest to l/d , where d is the average nuclear spacing (1.21 μ). If for each nucleus there were one unit of radiosensitivity λ and there were n_r cells with r nuclei in a sample of N cells, the surviving fraction would be

$$S = \sum_r n_r S_r / N, \quad (3)$$

where S_r is given by equation (1) and the summation is over all observed values of r .

Values of S have been calculated for a number of values of λD in equation (2) and they have been fitted to the observed surviving fractions ${}_0S$ at different doses D in the following way. $\log {}_0S$ has been plotted against $\log D$ on one piece of paper and $\log S$ against $\log \lambda D$ on a separate piece of transparent paper. The two superimposed sets of points were then made to lie as nearly as possible on the same curve by a shift of one set parallel to the $\log D$ axis. The magnitude of this shift was a measure of $\log \lambda$, since $\log \lambda D = \log \lambda + \log D$. The value of $\log \lambda$ for the best fit was found by the method of least squares. For cultures grown in the absence of gamma radiation a weight proportional to the square root of the number p_i of surviving cells (colonies) counted was assigned to each experimental point (the mean variance of replicate counts was $3p_i$) and the value of $\sum_i (\log {}_0S - \log S)^2$ found for different assumed values of λ . The minimum value of this sum, which corresponded to the best value of λ , was found by graphical interpolation. For cultures grown with gamma irradiation the variance of replicate counts increased with dose from $2p_i$ at zero dose to $9p_i$ at 20 kr. The weights assigned to points for different doses were adjusted accordingly and the rest of the fitting procedure carried out as already described.

If there were a systematic discrepancy between the shapes of the calculated curves of best fit and the observed ones, this would be expected to show up when the results of all experiments at each dose were pooled and the mean of the ratios ${}_0S/S$ plotted as a function of dose; Fig. 4 shows the results of this test. For 18 cultures grown in the absence of gamma radiation and for 9 grown under gamma irradiation the means of the ratios differed from unity by less than their standard deviations, indicating no significant discrepancy at any dose.

Some idea of the limits within which the average 'target' spacing could be fixed by the survival data alone was found by calculating surviving fractions for typical long (11μ) and short (2.5μ) populations with assumed 'target' spacings of $2 \times 1.2 \mu$ and $0.5 \times 1.2 \mu$. These fractions were then compared with those calculated for a spacing of 1.2μ ; the results are shown in the lower half of Fig. 4. In both cases the deviations from unity exceed the mean experimental ones, indicating that a spacing of 1.2μ fits the experimental data better than either of the alternative spacings.

Radiosensitivity of the targets

Having found the calculated curve of the best fit for each culture, the value of λ followed at once. For cultures grown in the absence of gamma radiation at 22° and 37° , the average value of λ was 0.349 ± 0.025 , whilst for cultures grown under gamma irradiation at 600 r./hr. at 22° , and 1000 r./hr. at 37° , it was 0.289 ± 0.040 . These values are significantly different ($P < 0.01$).

Lengths of bacterial segments

The number of segments into which each living cell was divided by visible 'waists' was noted when its length was measured under the microscope. The proportion of the population with 1, 2, 3, ..., segments varied from culture to culture, but the variations were not large and no correlation of the proportions with cultural conditions was evident. The length of the shortest cells in a culture was almost

Table 3. *Sizes of cell segments under different cultural conditions*

Average lengths are given for the whole population and for those cells which comprise the shortest 2 % of the population.

Cultural conditions			Average segment lengths for cells composed of			
			1 segment		2 segments	
Temp.	Population density (ml.)	Gamma radiation *	All cells (μ)	Shortest 2 % (μ)	All cells (μ)	Shortest 2 % (μ)
23°	10 ⁷ -10 ⁸	—	4.1	1.5	2.9	1.5
23°	10 ⁷ -10 ⁸	+	7	1.8	5	1.8
37°	10 ⁷	—	7	2.4	5	2.1
37°	10 ⁷	+	14	1.6	10	1.8
37°	4 × 10 ⁸	—	3.2	1.6	2.4	1.5
37°	4 × 10 ⁸	+	3.5	1.4	2.9	1.5

independent of the cultural conditions, in contrast with the large dependence shown by the average length. This is illustrated in Table 3 in which data for cultures grown under similar conditions have been pooled. Results for cells with one segment and two segments are separately classified, the figures for the shortest cells being the average for 3-5 cells which comprised the shortest 2 % of each class. Although the choice of the fraction 2 % was arbitrary, these results show that the smallest bacteria may be presumed to have lengths in the range 1.5-2 μ at 'birth'.

DISCUSSION

Brownell (1955) reported an approximate equality between the average number of radiosensitive units/cell and the average number of nuclei/cell for comparatively short cells (1-4 nuclei/cell) of *Escherichia coli* strain B/r from batch cultures in minimal medium. The values of radiosensitivity given by Brownell's data, namely, 0.45 and 0.50, may be compared with our value of 0.35.

For cultures growing under γ -rays the doses received by cells during one generation time were in the region of 10³ r., so only a very small % of cells should have failed to form colonies when plated (Fig. 1). Although the measured proportions of viable cells were not at variance with this expectation, the fact that the growth rate was not significantly decreased by γ -irradiation (Maclean & Munson, 1961) was the best evidence that radiation damage was not serious for the culture as a whole.

Our observations on cells treated with lysozyme indicate that one membrane normally encloses the contents of one segment and that cell division does not always take place as soon as the division of the cell membrane is complete. If there are transverse septa within segments they must be relatively weak, otherwise their presence would have been evident during the change of segment shape from rod to sphere.

It has been shown that the aggregation of chromatinic material in bacteria exposed to damaging agents such as ultraviolet radiation is dependent upon the salt concentration of the suspending medium (Whitfield & Murray, 1956). There are, however, diverse views about the significance of nuclear aggregation (Williams, 1959) so it would be premature to attempt to explain the role of gamma radiation in producing nuclear aggregation in *Escherichia coli* strain B/r. Whatever its cause,

nuclear aggregation in the γ -irradiated cells appeared to increase rather than to decrease the chance of surviving X-irradiation, so it may be presumed that undamaged nuclei can extricate themselves and initiate cell division. The absence of any transverse membrane within a segment would permit rapid diffusion of materials within the membrane and also perhaps a re-organization of aggregated nuclear material after the manner of fusion nuclei as envisaged by Bisset (1948).

The relative constancy in the length of the shortest segments of the living cells, grown under a variety of conditions (Table 3), shows that there was a preferred minimum length and that some cells of this length always arose despite a general inhibition of cell division in the population as a whole. The close correspondence between this minimum length (about 1.5μ), the length/nucleus given by staining (1.2μ) and the length occupied by one radiosensitive unit ($1-1.5 \mu$) is strong evidence that a radiosensitive unit contains one nucleus and is functionally almost identical with the smallest cell of *Escherichia coli* strain B/r which can exist. In order that it should function independently each unit must have at least one nucleus and the average one must contain less than two; otherwise the experimental and calculated survival data could not be reconciled.

The uniformity in the spacing of nuclei along cells of all lengths shows that nuclear division proceeded in step with cytoplasmic growth at a constant rate over a wide range of population densities at 37° (compare Katchman, Spoerl & Smith, 1955). On the other hand, different population densities had a marked effect on cell division, one pair of transverse membranes being formed on the average for each pair of nuclei at high population densities and for each 4 or 5 pairs of nuclei at low population densities. Thus the 'terminal stage' of cell division, namely, the laying down of transverse cell membranes followed by segmentation and separation of the daughter cells was much more sensitive to environmental changes than was the rate of production of nuclear and cytoplasmic materials. The appearance of 'giants' among X-irradiated mammalian cells (Puck & Marcus, 1956) implies a similar inhibition of cell division. Moreover, with *Escherichia coli* strain B/r the wide range of cell lengths within one culture indicates that individual bacteria differ from each other in respect of cell division, although for the culture as a whole the number of nuclei produced/cross-wall has a definite characteristic value. This characteristic value is dependent upon the three environmental factors; temperature, population density and gamma-radiation dose rate. Since the effect of one factor can be modified by either of the others, each probably affects the same process (Maclean & Munson, 1961). The evidence provided by the present experiments leads us to conclude that this process is the terminal stage of cell division and that it can be inhibited almost completely without quantitatively affecting the continued multiplication of other cellular components. For an individual cell, completion of division may be simply a matter of random chance, although statistically one could assign to it a certain probability/unit time. According to this view no fundamental distinction can be made between cells of different lengths in the same culture.

We gratefully acknowledge the helpful advice of Dr K. A. Bisset on the technique of nuclear staining. We are also indebted to Miss T. Alper and Dr N. E. Gillies for allowing us to see their experimental data about 'restoration' before publication, and to Miss P. A. Jeffery for very able technical assistance.

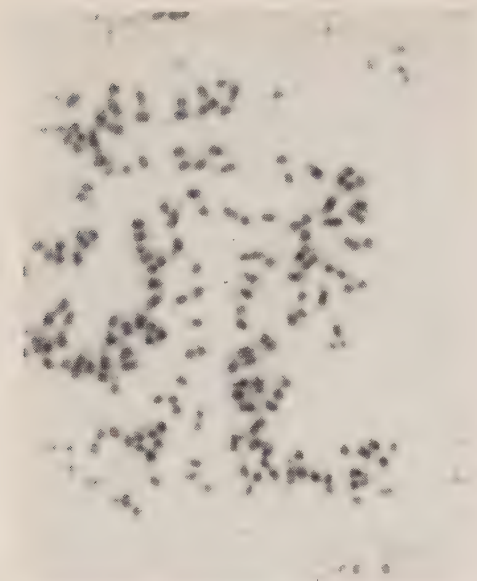


Fig. 1



Fig. 2

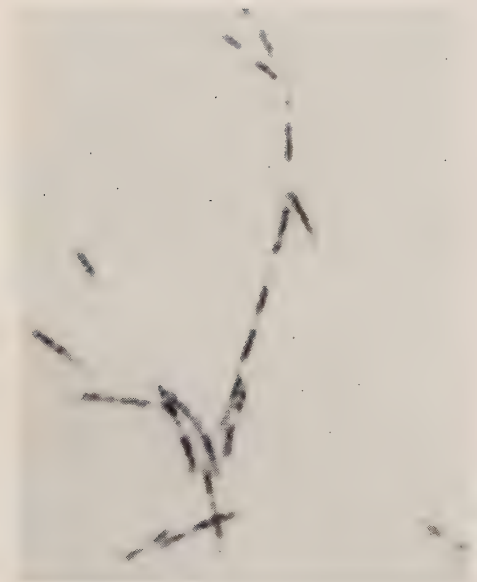


Fig. 3

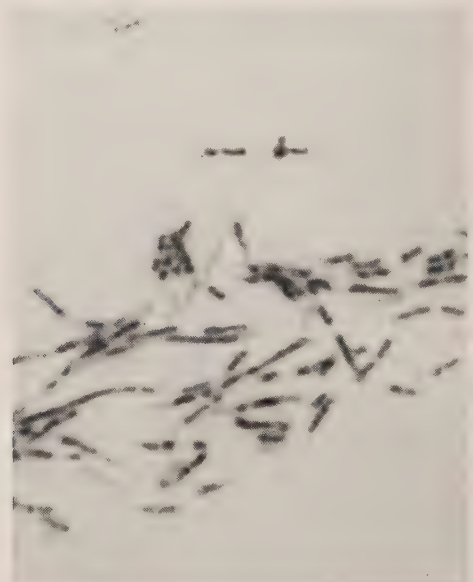


Fig. 4

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EXPLANATION OF PLATE 1

Escherichia coli strain B/r stained by HCl-Giemsa method and mounted in Canada balsam. Magnification, $\times 2520$.

Fig. 1. Cultivation at 22° at population density 7×10^8 /ml.; no gamma radiation. Cells had one or two nuclei only.

Fig. 2. Cultivated at 37° at population density 10^7 ; no gamma radiation. Cells with 4 and 8 nuclei were most common, nuclei being usually in close pairs or groups of four.

Fig. 3. Cultivated at 22° at population density 2×10^7 /ml. under gamma irradiation at 600 r/hr. Nuclear pattern much less regular than in fig. 2; there is some aggregation.

Fig. 4. Cultivated at 37° at population density 4×10^6 /ml. under gamma irradiation at 1000 r/hr. Nuclear pattern in longer cells very irregular; gross aggregation. Several cells appear to have no nuclei.

Imidazole Compounds Accumulated by Purine Mutants of *Neurospora crassa*

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SUMMARY

A procedure is given for the detection of imidazole compounds accumulated in the mycelium of adenine mutants of *Neurospora crassa*. Of five such compounds detected, four have been tentatively identified. The distribution of these imidazoles among the mutants investigated allows a correlation between the adenine loci and the steps of purine biosynthesis.

INTRODUCTION

Extensive enzymic studies with avian liver systems have led to the elucidation of the complete pathway of adenosine-5'-monophosphate biosynthesis (Buchanan, 1958-59). The seven reaction steps following imidazole ring closure are shown in Fig. 1. From studies of the corresponding reaction pathway in *Neurospora crassa*, Giles, Partridge & Nelson (1957) have presented evidence that E mutants (Table 1) are blocked in the conversion of inosinic acid to adenylosuccinic acid ribotide. F mutants

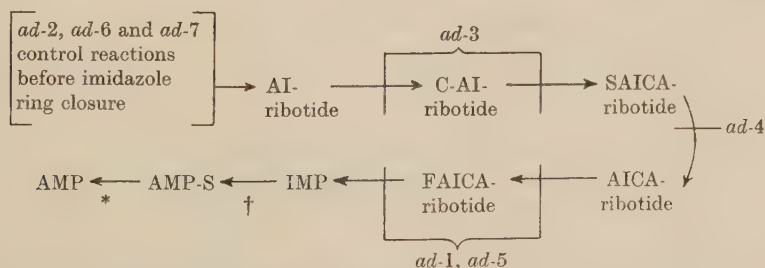


Fig. 1. Steps in purine biosynthesis controlled by adenine loci as judged from imidazole accumulation by mutants of *Neurospora crassa*.

Key: AMP, adenosine-5'-phosphate; AMP-S, adenylosuccinic acid ribotide; IMP, inosinic acid; FAICA-ribotide, 5-formamido-4-imidazolecarboxamide ribotide; AICA-ribotide, 5-amino-4-imidazolecarboxamide ribotide; SAICA-ribotide, 5-amino-4-imidazole-*N*-succinocarboxamide ribotide; CAI-ribotide, 5-amino-4-imidazolecarboxylic acid ribotide; AI-ribotide, 5-amino-imidazole ribotide.

* Giles *et al.* (1957) have shown that mutants at the *ad-4* locus are deficient in a de-acylase which splits both SAICA-ribotide and AMP-S.

† *ad-8* mutants were shown also by Giles *et al.* (1957) to be blocked in the conversion of IMP to AMP-S.

and mutant 44206 were also shown to lack activity for a bifunctional enzyme which catalysed the de-acylation of 5-amino-4-imidazole-*N*-succinocarboxamide ribotide and adenylosuccinic acid ribotide to 5-amino-4-imidazolecarboxamide ribotide

and adenosine-5'-phosphate, respectively. Dr T. French is reported (Buchanan, 1958-59) to have found in wild-type *N. crassa* all the purine biosynthetic enzymes present in the avian liver system. In the present work mutants representing several adenine loci of *N. crassa* were studied for their ability to accumulate imidazole compounds, intermediate in purine biosynthesis. All adenine mutants found to date have been allocated to eight loci (Barratt, Newmeyer, Perkins & Garnjobst, 1954; Giles *et al.* 1957); these are summarized in Table 1.

Table 1. *Summary of mutants of Neurospora crassa assigned to the eight adenine loci*

Locus	Mutants
<i>ad-1</i>	3254*
<i>ad-2</i>	70004, 27663, 20705
<i>ad-3</i>	35203, 38701, 38709, 45601, 68306, A†, B
<i>ad-4</i>	44206, 44415, F
<i>ad-5</i>	71104, J
<i>ad-7</i>	28610
<i>ad-6</i>	44411
<i>ad-8</i>	E

* Mutants with numerical designations came from the early mutant searches of Beadle & Tatum (1945).

† Letter designations were given by Giles *et al.* (1957) to groups of mutants related by heterokaryon complementation tests.

METHODS

Extraction of mycelia

The cultures of *Neurospora crassa* used as stocks, or as a source of conidial inocula, were grown in test tubes containing a complete medium described by Horowitz (1947). To obtain large quantities of mycelium, mutants were grown in carboys containing 10 l. of minimal medium (Beadle & Tatum, 1945) supplemented with limiting amounts of adenine sulphate (usually 30 μ g./ml.). All mutants were grown at 25°, except the temperature sensitive strains (44206, 44415, 70004) which were grown at 35°. After 3-4 days of growth under forced aeration the mycelia were harvested by pouring the contents of the carboys through cheesecloth. The mycelial mats were placed in a Waring Blender and boiling water was added (about 1 l./100 g. wet mycelium). After disintegration the extracts were filtered through Whatman no. 1 filter paper on a Buchner funnel. The filtrates were then lyophilized overnight, leaving thick syrupy extracts in the flasks. Five to 10 ml. of water were sufficient to take up the extract from 100 g. mycelium. Residual material were separated by centrifugation and washed several times with 1 ml. portions of water which were added to the soluble fraction. The solutions contained all the imidazoles. The residues were discarded.

Detection of accumulated imidazoles

The redissolved extracts were spotted directly, or with dilution, on Whatman no. 1 paper and resolved by ascending chromatography in a variety of solvents. Isopropanol, water, and conc. aqueous NH_4OH (sp. gr. 0.880) in the volume ratios 70:20:10 and 70:40:10 were the most useful systems. After drying, the chromatograms were sprayed very lightly with diazosulphonic acid reagent (Ames & Mitchell,

1952), and again very lightly with 5% (w/v) aqueous Na_2CO_3 solution. By this procedure five distinct imidazole compounds not present in wild-type mycelium were detected in extracts from the mycelia of the mutant strains.

Isolation of Compounds I and II (Table 3)

To 14 ml. of the water soluble extract of 135 g. wet weight of mutant 44206 mycelium (see Methods) an equal volume of methanol was added. A precipitate was formed which was washed with 50% (w/v) methanol in water. The soluble fractions, containing virtually all of Compounds I and II, were combined to a total volume of 23.5 ml.

A Dowex exchange resin (1-X 2, 200–400 mesh), from which the fines had been removed by repeated washings, was equilibrated with 2 M-formic acid. This was placed in a column 2.2 cm. in diam. to a volume of 170 cm.³. Water at 3.5° was circulated through a jacket which surrounded the column. The flow rate of solvent was kept at about one drop every 15 sec. by maintaining the system under controlled positive pressure. A mixing vessel of 125 ml. capacity was included in the system to allow gradient elution according to the procedure of Thompson (1955). The column was first washed with distilled water and then the sample was added. For elution, the series of solutions listed in Table 2 were used in the order given. Successive solutions were added only after the pH value of eluted fractions did not change measurably. By using an automatic fraction collector, 5–10 ml. portions were collected. Compound II began to elute at pH 1.9 (solution 7); it came off the column in a total volume of 273 ml. After an additional 135 ml. of eluent had passed through, compound I began to appear and came through in the next 82 ml.

Table 2. *Solutions used for the serial elution of chromatographic columns*

No.	Solution	Molarity of NH_4^+	Molarity of anion	Measured pH value
1	Distilled water	.	.	.
2	Ammonium acetate buffer	0.4	1.2	4.28
3	Ammonium acetate buffer	0.4	1.0	4.40
4	Ammonium formate buffer	0.4	1.1	3.28
5	Acetic acid	.	1.0	2.53
6	Formic acid	.	1.0	1.97
7	Formic acid	.	6.0	1.17

Separation of Compounds III and IV (Table 3)

An extract of mutant 45601 containing both Compounds III and IV was subjected to a chromatographic procedure similar to that described above. Compound III was eluted by water very close to the front, and Compound IV came off soon after. Though separation was achieved, both fractions were impure. However, they were satisfactory for chromatographic comparisons.

Abbreviations

The following abbreviations, modified from Buchanan (1958–59), are used: AMP, adenosine-5'-phosphate; AMP-S, adenylosuccinic acid ribotide; IMP, inosinic

acid; FAICA-ribotide, 5-formamido-4-imidazolecarboxamide ribotide; AICA-ribotide, 5-amino-4-imidazolecarboxamide ribotide; SAICA-ribotide, 5-amino-4-imidazole-*N*-succinocarboxamide ribotide; CAI-ribotide, 5-amino-4-imidazolecarboxylic acid ribotide; AI-ribotide, 5-amino-imidazole ribotide.

RESULTS

The colour reactions and distribution of the five detected imidazoles among the mutants are detailed in Tables 3 and 4.

Table 3. *Colour reactions of accumulated compounds*

		Compounds				
		I	II	III	IV	V
Colour development	Before Na ₂ CO ₃	Red-orange	Bright orange	Bright yellow	No colour	Yellow
	After Na ₂ CO ₃	Fades to grey	Fades to grey	Grey spots—fades	Red	Blue
Probable identity		SAICA-ribotide	SAICA-riboside	AI-riboside	Unknown	AICA-riboside

Table 4. *Distribution of accumulated imidazoles*

		Compounds				
Locus	Mutants	I	II	III	IV	V
<i>ad-1</i>	3254	—	+	—	—	+
<i>ad-2</i>	70004	—	—	—	—	—
	27663	—	—	—	—	—
	20705	—	—	—	—	—
<i>ad-3</i>	35203	—	—	+	+	—
	38701	—	—	+	+	—
	38709	—	—	+	+	—
	45601	—	—	+	+	—
<i>ad-4</i>	44206	+	+	—	±	—
<i>ad-5</i>	71104	—	+	—	±	+
<i>ad-6</i>	28610	—	—	—	—	—
<i>ad-7</i>	44411	—	—	—	—	—

The following designations were used: +, compound present in mycelial extract; ±, compound present, but in low concentration; —, compound not detected.

Characterization

Compound I at pH 7 had an ultraviolet absorption peak at 269 mμ. By the method of Mejbaum (1939) it was shown to contain a pentose moiety. A sample of SAICA-ribotide generously provided by Dr T. French proved to be identical with Compound I, both in colour development and chromatographic mobility. When Compound I and the known SAICA-ribotide were hydrolysed by alkaline phosphatase each formed a product identical on chromatograms with Compound II.

Compound II at pH 7 absorbed maximally at 268 mμ, and was also shown to contain a pentose moiety. By using the procedure of Allen (1940) it was proved to lack a phosphate group. On hydrolysis at 105° with conc. HCl, for 15 hr. in a sealed

tube, Compound II yielded aspartic acid and glycine. The presence of aspartic acid on hydrolysis, the shape of the ultraviolet absorption curve, and position of the absorption maximum compared well with the properties reported for SAICA-ribotide (Gots & Gollub, 1957; Lukens & Buchanan, 1959*a*). The phosphate determination and the data from hydrolysis with alkaline phosphatase further showed that Compound II was SAICA-riboside.

Compound III formed an orange-red Bratton-Marshall reaction product (Bratton & Marshall, 1939) which absorbed maximally at 500–502 $m\mu$. No absorption maximum in the ultraviolet region was detected. That Compound III failed to bind to an anion exchange resin argues against the presence of carboxyl or phosphate group. The Bratton-Marshall product of AI-ribotide is reported to be salmon-orange and to absorb maximally at 500 $m\mu$ (Lukens & Buchanan, 1959*b*). Also this compound is reported to have no ultraviolet absorption maximum above 210 $m\mu$. All the properties observed for Compound III are consistent with its being AI-riboside.

The Bratton-Marshall reaction product of Compound IV was orange-red and absorbed maximally at 534–538 $m\mu$. This corresponds to none of the maxima reported for the imidazoles involved in purine biosynthesis. However, since the concentration of this compound appeared to decrease as more care was taken in preparation of extracts, it seems likely that Compound IV was a reaction product of Compound III.

Ames & Mitchell (1952) reported that, of 16 compounds tested by their diazotization procedure, only 5-amino-4-imidazolecarboxamide (AICA) gave a blue reaction product. Subsequently AICA-ribotide was observed by the present author to give this blue colour. Compound V gave a colour reaction identical with that of the aglycone and AICA-ribotide. Chromatographic evidence suggested that Compound V was either AICA or AICA-riboside.

DISCUSSION

Knowing the distribution and probable identity of the detected imidazole compounds it is possible to correlate the adenine loci and the steps of purine biosynthesis (Fig. 1). Since *ad-2*, *ad-6* and *ad-7* mutants of *Neurospora crassa* can use IMP or hypoxanthine in place of adenine as a growth supplement (Mitchell & Houlahan, 1946) and yet accumulate no imidazole compounds, they can be assigned to steps preceding imidazole ring closure. This conclusion is in accord with an observation of Mitchell & Houlahan (1946). All *ad-3* mutants accumulate a distinctive purple pigment in their growth media. The purple pigment, which seems to have associated with it a 305 $m\mu$ ultraviolet absorption maximum, is probably a reaction product of Compound III. It was also shown by these authors that the double mutants of 35203 (*ad-3*) in conjunction with *ad-2*, *ad-6* and *ad-7* mutants, accumulate no purple pigment. The double mutant 35203, 44206 (*ad-4*) does accumulate the pigment. This again places the *ad-2*, *ad-6* and *ad-7* block before, and the *ad-4* block after, the reaction controlled by *ad-3*.

Colourless solutions of SAICA-riboside (Compound II) will turn red in time, especially at low pH values or on exposure to air. The reddish appearance of mutant 44206 (*ad-4*) mycelia is probably due to this effect. The percentage yield of SAICA-riboside and SAICA-ribotide in the dried mycelia of mutant 44026 can be calculated

from the observed optical densities at the ultraviolet absorption maxima and the extinction coefficient given by Lukens & Buchanan (1959*a*). The yields of the riboside and ribotide were found to be 2.1 and 0.023 %, respectively, of the dry weight of its mycelia.

The two intermediates CAI-ribotide and FAICA-ribotide and the corresponding ribosides, known to be the least stable of the imidazoles, were not detected in any extracts.

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The Pure Culture of *Physarum polycephalum* on a Partially Defined Soluble Medium

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SUMMARY

A wild strain of the multinucleate plasmodial myxomycete *Physarum polycephalum* was isolated in pure culture and grown on a medium consisting of 1 % (w/v) Tryptone, 1 % (w/v) glucose, 0.15 % (w/v) yeast extract, 0.3 % (w/v) CaCO_3 , inorganic salts and a small amount of chick embryo extract. The organism may be grown with this medium either as a single large plasmodium on surface culture, or as a suspension of tiny plasmodia in submerged culture. From an initial inoculum of 1 ml. of a 3-day culture, the average plasmodial yield in a submerged culture was about 80 mg. dry weight/20 ml. medium at 72 hr. Growth occurred only in the presence of small amounts of an unidentified factor which was present particularly in chick embryo extract and foetal calf serum. An isolate of *P. polycephalum* was grown continuously on this medium for over four years without an appreciable decrease in growth rate. Under proper conditions a suspension of tiny plasmodia from shaken culture will fuse to form a single large surface plasmodium which exhibits synchronous mitosis.

INTRODUCTION

The myxomycete *Physarum polycephalum* possesses a number of characteristics which recommend it for studies of cellular activities: (i) mitoses are essentially synchronous in the multinucleate plasmodium (Howard, 1932); (ii) the plasmodia are sufficiently large to permit the correlation of changes in cell structure with changes in chemical content, since samples may be taken simultaneously and sequentially from a single plasmodium with a minimum disturbance to the organism; (iii) the stages of proliferation and differentiation (sporulation) are distinct and separable and may be controlled by the investigators (Daniel & Rusch, 1958).

Although the myxomycetes have been recognized for over a century (Schweinitz, 1822) most of the studies on these organisms have been morphological and taxonomic in nature. This limited study may be attributed to a general unawareness about this group of organisms but particularly to a lack of adequate methods of laboratory cultivation. Previous studies were conducted with impure cultures grown on oats or other natural substrates (Howard, 1931, 1932; Camp, 1936). Cohen (1939, 1941) and Sobels (1950) reported the isolation of pure cultures which grew on autoclaved yeast or oats, or in the presence of another organism, and Hok (1954) obtained growth on autolysed yeast preparations. Although pure cultures were apparently obtained, sustained growth was achieved only with insoluble natural media unsuitable for nutritional and biochemical investigations. This paper

presents methods for the growth of *Physarum* on a soluble medium free from other organisms (axenic culture) and describes the growth characteristics of the cultures. The morphological features of the organism grown under these conditions are described elsewhere (Guttes, Guttes & Rusch, 1961).

METHODS

Isolation. The culture of *Physarum polycephalum* used was isolated by permitting a plasmodium, reconstituted from the sclerotial stage (kindly furnished by Professor M. P. Backus, Department of Botany, University of Wisconsin) to migrate across sterile phosphate agar (pH 4.3) at 20°. Fragments of the arborescent plasmodium were cut out with a sterile knife, transferred to a similar sterile agar plate, and this procedure repeated four or five times with each isolate. Only plasmodial branches which had undergone maximum migration without intersecting their own paths were used. Such portions could be selected since plasmodia deposited an easily visible path of slime. To detect the presence of contaminating organisms, the plasmodia were then allowed to migrate on nutrient agar plates at pH 5 and at pH 6, and these fragments were transferred to these media a second time. The plasmodia were next transferred to nutrient agar plates (pH 5) streaked with sterile rolled oats and whole yeast, and incubated at 20°. The nutrient agar plates from which these plasmodia were removed were then incubated at 20° and 37°, and the migration path inspected for the appearance of colonies of microbial contaminants. When these plates showed no contaminants the plasmodia transferred from them were provisionally assumed to be pure and were transferred from the yeast + oats nutrient agar plates to Erlenmeyer flasks containing sterile oats, and incubated at 20°. These flasks were prepared by autoclaving 2 g. dry cereal-grade oats/500 ml. flask for 1 hr. at 121°. After being cooled, the oats were moistened with about 2 ml. sterile distilled water/g. oats. The transferred plasmodia were very small, and, because of this, growth on the moist oats could be observed only after 3–5 days. Transfers were made by loop, and care was taken to minimize damage to the fragments. Growth on this medium was rapid and profuse. After several transfers the organism was tested for contamination by dispersing plasmodial fragments of different ages in shaken and deep-tube static culture, by streaking on agar plates and by stab cultures at 21°, 28° and 37°, at pH 4 and 6. The media used were oat agar and a peptone Tryptone beef-extract yeast-extract agar, with and without glucose. After various periods of incubation the cultures were examined microscopically for evidence of contamination. In addition, direct microscopic examination of stained and unstained *Physarum* cultures was made. Samples of old degenerated cultures were also examined by the same procedures. The possibility that *P. polycephalum* may suppress the growth of contaminants by phagocytosis or by its reported antibiotic production (Sobels, 1950) is minimized, since *Physarum* degenerates in static submerged liquid culture at pH 6 or above. Although subsequent tests showed that penicillin and streptomycin, at 1000 and 100 units/ml., respectively, had little effect on the organism grown under the conditions employed, these antibiotics were not used for the initial isolation of the cultures.

Culture medium. Pure cultures of *Physarum polycephalum* were maintained on sterile rolled oats, as described in a preceding section, until a soluble medium was devised. The composition of the medium finally adopted for routine culture is given in Table 1.

Table 1. *Complete growth medium*

Component	Concentration (g./100 ml. medium)	Component	Concentration (g./100 ml. medium)
Tryptone (Difco)	1.0	MnCl ₂ .4H ₂ O	0.0084
Yeast extract (Difco)	0.15	ZnSO ₄ .7H ₂ O	0.0034
Glucose, anhydrous	1.0	Citric acid.H ₂ O	0.048
KH ₂ PO ₄	0.20	HCl, concentrated	0.006 ml.
CaCl ₂ .2H ₂ O	0.06	Distilled water	to 100 ml.
MgSO ₄ .7H ₂ O	0.06	CaCO ₃	0.30
FeCl ₂ .4H ₂ O	0.006	Chick embryo extract*	1.5 ml.

* Difco ampoule containing 2 ml. of a lyophilized 50 % extract reconstituted with 8.3 ml. distilled H₂O.

The following stock solutions were diluted to give the concentrations listed in Table 1: glucose, 20.0 % (w/v); Tryptone, 10.0 % (w/v); yeast extract, 4.0 % (w/v); KH₂PO₄, 4.0 % (w/v). All inorganic salts, as well as citric acid and HCl but not CaCO₃, were dissolved together so that 3.0 ml. of the concentrated solution in 100 ml. medium gave the final concentrations shown in Table 1. The above components were combined and autoclaved for 20 min. at 121°. A very pale yellow colour is characteristic of the concentrated salt solution freshly prepared with pure ferrous chloride. However, salt solutions which develop a marked increase in yellow colour, indicating the oxidation of ferrous to ferric iron, should not be used, since the organism requires ferrous iron. The stock solutions with a few drops of toluene added were stored in a refrigerator.

Calcium carbonate was autoclaved as a 10 % (w/v) suspension in distilled water for 1 hr. at 121°, cooled, and then added to this cooled medium. The pH value of the autoclaved suspension of CaCO₃ was approximately 8 to 8.5. Preparations of CaCO₃ of higher pH value made the final medium exceed the optimal value of pH 5 and were avoided.

The lyophilized embryo extract (Difco) was reconstituted with 8.3 ml. sterile distilled water/ampoule (equivalent to 2 ml. of 50 % freshly prepared non-lyophilized extract) and was added to the solution with a sterile syringe after the addition of CaCO₃. The complete medium, buffered at pH 5.0 with CaCO₃, was then dispensed in the desired sterile culture vessels, into which the organism was then inoculated. Care was taken to keep the CaCO₃ suspended while the medium was being dispensed.

Submerged agitated culture. Submerged cultures were prepared by allowing a plasmodial fragment to migrate from the wetted bottom of a tilted 500 ml. Erlenmeyer flask containing 20 ml. of growth medium, on to the liquid surface. The flask was then placed upright to float the piece of plasmodium and incubated without agitation for 48–72 hr. Care was taken not to submerge the organism, since this caused degeneration in static cultures. After the plasmodium had grown for about 48 hr., the flask was agitated at 170 reciprocations/min. with a stroke length of 1½ in. and maintained at 21.5° ± 1°. The organism fragmented into tiny plasmodia (micro-

plasmodia) under such conditions and was maintained in shaken culture by serial transfer. Since growth may be retarded by prolonged exposure to ordinary room lighting, the cultures were kept in the dark except during periods of examination.

After several transfers satisfactory growth was obtained and a constant culture cycle established. Plasmodia from such cultures tended to adhere to the walls of the flask and had to be shaken down periodically to insure reproducible growth rates. Shaken cultures were routinely maintained in 500 ml. Erlenmeyer flasks containing 20 ml. medium. One ml. of a 72 hr. culture containing the equivalent of about 450 μ g. plasmodial nitrogen, inoculated into 20 ml. medium, produced a culture of similar plasmodial concentration after 72 hr. of growth.

Surface culture. Surface cultures of a single plasmodium were grown on filter paper supported at the surface of the liquid medium on glass beads, either in Petri dishes or in Erlenmeyer flasks. Cultures were inoculated either with single fragments cut from other surface plasmodia or with suspensions of 2- to 3-day shaken cultures. Samples (1-2 ml.) of the latter were pipetted directly on to a dry filter paper (Whatman no. 40) and allowed for several hours to fuse into a single plasmodium before the medium was added below the filter paper. Earlier addition of the medium retarded fusion. About 13 ml. medium were required just to cover a layer of glass beads (about 3.5 mm. diam.) in a 9 cm. diam. Petri dish. When a larger surface plasmodium was desired, the culture was first centrifuged, resuspended in a volume of CaCO_3 -buffered salts medium (pH 5) equal to the packed plasmodial volume, and dispersed in a thin layer on the filter paper. (This medium was prepared by diluting 3.0 ml. inorganic salts + citric acid + HCl concentrate to 100 ml. with H_2O , autoclaving, and buffering with 2 ml. sterile 10% (w/v) CaCO_3 suspension; see Medium, Table 1.) Surface cultures carried in Petri dishes could not be used for the prolonged maintenance of the *Physarum* in pure culture because of their susceptibility to contamination, but such cultures could be maintained in 500 ml. Erlenmeyer flasks.

Agar cultures of the organism were prepared by autoclaving dry granular agar evenly distributed over the bottom of the desired culture vessel for 1 hr. and, after cooling, adding 5.7 ml. medium/g. agar. These cultures were inoculated with loop-transferred fragments of plasmodium or with suspensions of microplasmodia from agitated cultures.

Stock cultures. Cultures were maintained routinely in shaken flasks and on the surface of granular agar in Erlenmeyer flasks. Each of two cultures was inoculated into triplicate cultures. Unopened cultures from two previous transfers were retained as reserve inocula. The shaken cultures were transferred every 3 days, and the surface cultures every 6-7 days.

The isolates now in use have been maintained continuously in various types of culture for more than 4 years and are periodically re-examined for purity. Particular attention must be paid to aseptic technique, since the relatively slow multiplication rate of *Physarum* enables many microbial contaminants to establish themselves without being readily detected for a number of transfers.

In addition to the original isolate, a few specimens of *Physarum polycephalum* obtained from other sources have also been isolated, and, apart from some characteristic differences, all have grown under the conditions described in this paper.

Analytical procedures. The growth of *Physarum polycephalum* was estimated by the measurement of the total trichloroacetic acid-precipitable nitrogen and of the dry weight. Nitrogen was estimated by the microKjeldahl method of Johnson (1941). Centrifuged plasmodia were suspended in a small volume of distilled water and precipitated with a volume of 8% (v/v) trichloroacetic acid (TCA) in acetone (TCA + acetone; 8.0 ml. of 100% (w/v) TCA diluted to 100 ml. with acetone) equal to the volume of the plasmodial suspension. After standing overnight at 5°, the suspension was centrifuged, the clear supernatant fluid saved for determination of the yellow plasmodial pigment, and the precipitate suspended and dissolved in 0.3 N-NaOH, samples of which were used for nitrogen analysis. Small amounts of the mucoprotein-like substances present in the medium of cultures older than 2.5 days and insoluble in TCA + acetone were unavoidably included in the analysis of these samples. Dry weight was determined as the weight of residue of washed plasmodia dried for 24 hr. at 115°.

The amount of yellow pigments was determined by reading suitable dilutions of the TCA + acetone supernatant fluid at 415 m μ in a cell of 1 cm. light path (E_{415}) in the Beckman DU spectrophotometer. The results were expressed as total absorption/20 ml. shaken culture or of a single 2 g. oat culture. The pigment has absorption maxima at approximately 385 m μ in dilute aqueous alkali and at approximately 415 m μ in aqueous TCA + acetone. The nature of the yellow pigments is under investigation by Professor F. M. Strong and associates (Biochemistry Department, University of Wisconsin; see C. F. Dresden, 1959).

Hydrogen-ion concentrations were determined with the Beckman model G pH meter.

RESULTS

Preliminary studies demonstrated that submerged rather than surface culture of *Physarum polycephalum* was the more useful form for determining nutritional and growth characteristics. In submerged culture growth was more rapid, more readily measured, and was easier to maintain free from contamination. This type of culture was also basically more useful, since the very small plasmodia so obtained readily fused, when pipetted on to filter paper, to yield large plasmodia suitable for study of synchronous growth.

Submerged agitated culture

Typical growth curves obtained in submerged culture for two different isolates with the Tryptone glucose medium are given in Fig. 1. The average plasmodial yield from 100 ml. medium at 3–4 days was equiv. to 0.42 g. dry weight, with an average microKjeldahl-N value of 8.7% and a dry weight of 18.7%.

To determine the optimal concentrations of glucose and Tryptone for growth, the amounts of these components were varied independently of each other in the basal medium (Fig. 2). The results indicated that 1.0% (w/v) of each was the most suitable for routine use. The small increase in yield achieved by increasing either one or both components to 1.5% (w/v) did not warrant the relatively large (50%) increase in amount of substrate. Glucose and Tryptone each at 2.0% (w/v) depressed yields considerably even when the optimal pH 5 was maintained. In the absence of glucose the maximal yield was 29% of that obtained with 1% (w/v) glucose. The change in slope of the variable-Tryptone curve suggests that Tryptone

may also serve as a precursor of products in addition to those required for plasmodial growth. The appearance of a viscous acid and 'Sevag-precipitable' product in the medium of cultures older than 60 hr. containing 1% (w/v) or more Tryptone supports this possibility. (An ethanol-insoluble fraction isolated from Sevag-precipitated culture filtrates was found by Professor M. Heidelberger, private communication, to be related to certain groups of pneumococcal capsular antigens.) Tryptone could be replaced by enzymic (Difco) or acid (General Biochemicals, Inc.) casein hydrolysates, peptone (Difco), or protone (Difco).

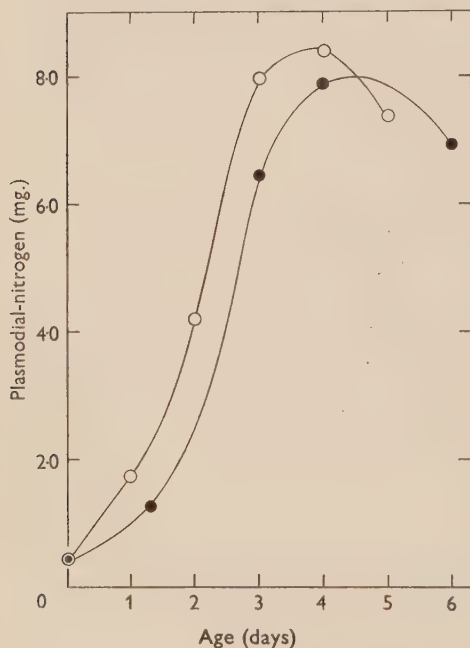


Fig. 1

Fig. 1. Growth of *Physarum polycephalum* in submerged culture on a semi-defined medium. —○—, —●—, represent the growth of isolates made 2 years apart from the same sclerotium. Cultures contained 20 ml. medium and 1.0 ml. inoculum/500 ml. Erlenmeyer flask and were incubated at 21–22°, agitated at 170 reciprocations/min. Plasmodial-N represents the average microKjeldahl-N of duplicate 21 ml. cultures. —●—, 4-day inoculum; —○—, 3-day inoculum.

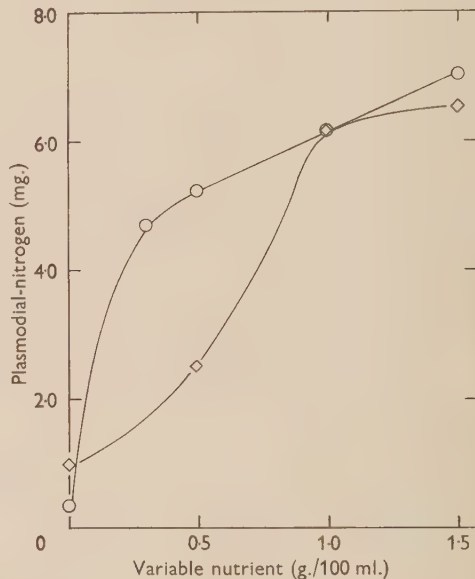


Fig. 2

Fig. 2. The effect of glucose and Tryptone concentration on the growth of *Physarum polycephalum*. —◇—, Tryptone, 1.0 g./100 ml. medium (Table 1), glucose concentration varied; —○—, glucose, 1.0 g./100 ml. medium, Tryptone concentration varied. Cultures contained 20 ml. medium and 1.0 ml. (equiv. 400 µg.-N) of a 4-day inoculum/500 ml. Erlenmeyer flask, harvested after 4 days growth in submerged culture at 21–22° and 170 recip./min.; plasmodial-N represents the average microKjeldahl-N of duplicate 21 ml. cultures.

The medium listed in Table 1 failed to support growth in the absence of chick embryo extract. The results in Fig. 3 show this requirement and clearly indicate that no growth occurred without embryo extract; the small yield obtained was essentially equivalent to the inoculum added. The flattening of the curve at maximum growth suggests that the active fraction of embryo extract was a micro-nutrient and not a gross nitrogenous source. The reconstituted embryo extract

listed in Table 1 contained about 0.6 mg. N/ml., while the complete medium contained about 1.7 mg. N/ml. Neither the pH value nor the concentrations of salts, yeast extract, glucose or Tryptone were growth-limiting in these experiments.

Of a large number of natural products and pure compounds tested, only chick embryo extract, foetal calf serum, and foetal calf erythrocyte haemolysate showed high growth-promoting activity. Less activity was found in chick and beef sera and in human umbilical cord serum. Although whole yeast and oat flakes also supported growth, various extracts of these materials were inactive.

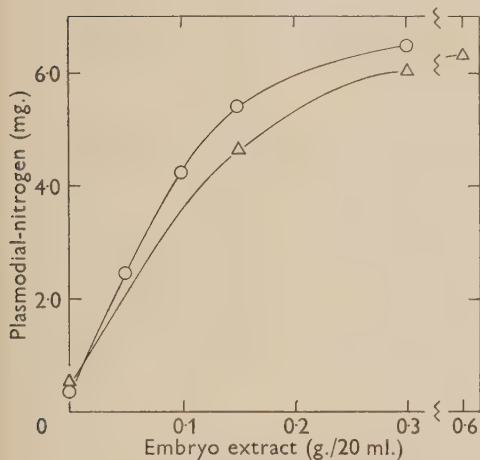


Fig. 3

Fig. 3. Embryo extract requirement for the growth of *Physarum polycephalum*. —○—, submerged cultures, 20 ml. medium/500 ml. Erlenmeyer flask, 1.0 ml. (equiv. 350 $\mu\text{g. N}$), 4-day inoculum; cultures harvested after 4 days incubation at 21–22° and 170 recip./min. —△—, surface cultures, 20 ml. medium absorbed by 3 g. agar (Difco, granular), dry-autoclaved in 500 ml. Erlenmeyer flasks, loop-inoculated with a small plasmodial fragment (equiv. about 470 $\mu\text{g. N}$)/flask; harvested after 10 days static incubation at 21–22° by floating plasmodium off agar surface. Chick embryo extract prepared in manner described for medium; plasmodial-N represents the average microKjeldahl-N of duplicate 21 ml. cultures.

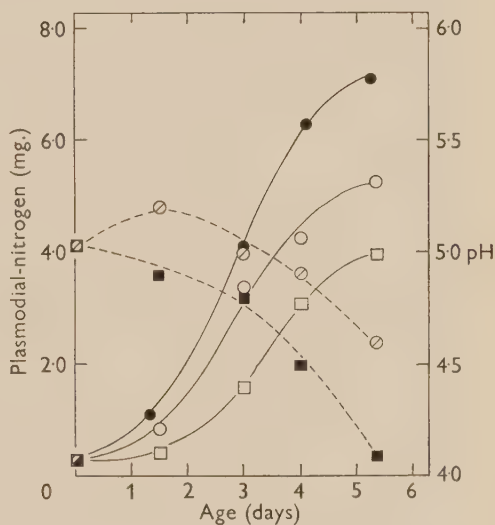


Fig. 4

Fig. 4. Effect of CaCO_3 and phosphate buffers on pH value of culture and plasmodial yield. All cultures contained 50 ml. medium/500 ml. flask and 2.5 ml. inoculum (equiv. 300 $\mu\text{g. N}$)/ml. and were incubated at 21–22° at 170 recip./min.; plasmodial-N represents the average microKjeldahl-N of duplicate 21 ml. cultures. —●—, N for 0.3% CaCO_3 buffer; —○—, N for 0.2% CaCO_3 buffer; ---○---, pH value for 0.2% CaCO_3 buffer; —□—, N for cultures buffered with KH_2PO_4 (adjusted pH 5) and added to give a medium concentration equivalent to 1.5% (w/v) KH_2PO_4 , CaCO_3 omitted. ---■---, pH value for phosphate-buffered cultures; sterile phosphate buffer added to medium after autoclaving.

Under otherwise optimum conditions, maximum growth was obtained when the soluble medium was maintained at pH 5 with calcium carbonate. The acid production accompanying growth and the necessity of using a buffer to obtain a maximum yield are shown in Fig. 4. The smaller buffering capacity of the phosphate at pH 5, as compared with that of 0.2% (w/v) CaCO_3 , permitted a steady decrease

in pH value which resulted in a 24% decrease in growth. When the amount of calcium carbonate was increased from 0.2 to 0.3%, the culture remained at pH 5 for 5 days (not shown in Fig. 4) and a yield of 3.55 mg. N/20 ml. was obtained. Since the optimum pH value of 5 was maintained well past the time of peak yield (3-4 days), cessation of net growth resulted either from a depletion of nutrients or from an increase in growth-inhibiting metabolites.

With citric acid buffer (0.5%, w/v, adjusted to pH 5) there was only 49% as much growth as with 0.3% (w/v) CaCO_3 . Acetic acid buffer (0.5%, w/v, adjusted to pH 5) inhibited growth completely. Potassium phosphate buffers at pH 4 and 6.8 supported only 30 and 2%, respectively, as much growth as was obtained with the same buffer at pH 5. When cultures were maintained on a medium buffered with 1.5% (w/v) KH_2PO_4 adjusted to pH 5, and free from added calcium, a requirement for Ca^{++} could be demonstrated (Fig. 5). The concentration of magnesium present in the growth medium (Table 1) did not replace Ca^{++} .

The growth behaviour as a function of culture volume is shown in Fig. 6. The yield was substantially improved by the better aeration provided by the smallest (20 ml.) volume. However, the interpretation is complicated for a coenocytic organism since the growth rate may also be influenced by the degree to which plasmodial fragmentation is affected by agitation. In addition to the effect of culture volume, the rate and type of agitation obtained on various shakers also influenced both aeration and fragment size. The extent to which the mechanical characteristics of these shakers contributed to differences in growth rate is being examined. Submerged cultures grown at atmospheric pressure under nitrogen showed no net growth.

Decreasing the size of the inoculum delayed the time required to reach the peak yield. As summarized in Table 2, the use of an inoculum equivalent to 450 $\mu\text{g.}$ -N resulted in the most rapid average duplication time ($t_{av.}$) and did not unduly prolong the time (a) of peak yield (y). A relatively small volume of inoculum also decreased the amount of culture fluid carried with each transfer.

The data of Table 3 summarize the relation between duplication time and pigment formation with this medium. From a comparison of the calculated N/pig. values tabulated against duplication times observed directly from the nitrogen curves, it is apparent that during the period of most rapid growth (0.5 ml. inoculum, at 0-28 hr.) pigment formation was relatively depressed. This was followed by a period of decline in growth rate, during which pigment formation abruptly increased. When the growth rate was constant but somewhat slower than the maximum (2.5 ml. inoculum, 0-28 hr.) the N/pig. ratio remained approximately constant and was followed after 46 hr. by a period during which growth declined and pigment formation rose rapidly. In each case the relative plasmodial pigment content was lowest (highest N/pig. ratio) at approximately the time of most rapid growth. The N/pig. ratio appeared to remain constant only when the duplication time was also constant. Studies in progress in this laboratory on the properties of the pigment indicate that the *in vitro* formation of a leuco form under reducing conditions may also occur *in vivo*. The N/pig. ratio may then reflect either the synthesis or oxidation-reduction ratio of the pigment.

The tendency of non-growing or slowly-growing plasmodia to accumulate pigment is a useful measure of the capacity of a medium to support growth. Fig. 7 shows the

data of Fig. 2 plotted as a function of pigment content. The increasing yields correlate with increasing N/pig. ratios but not necessarily with increased growth efficiency (g. plasmodium/g. substrates). Oat-grown plasmodia also continued to produce pigment (Fig. 8) for a considerable period after the termination of growth at 6 days as indicated by the increasing N/pig. ratio.

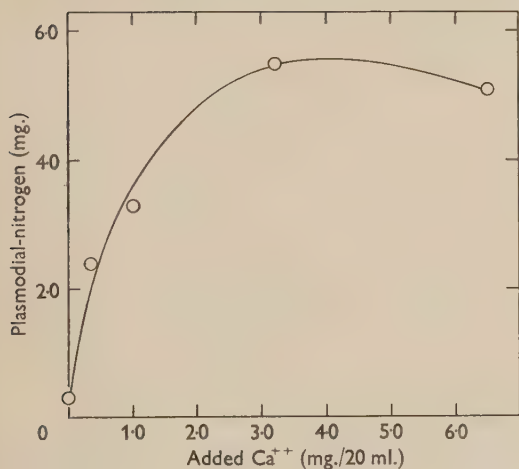


Fig. 5

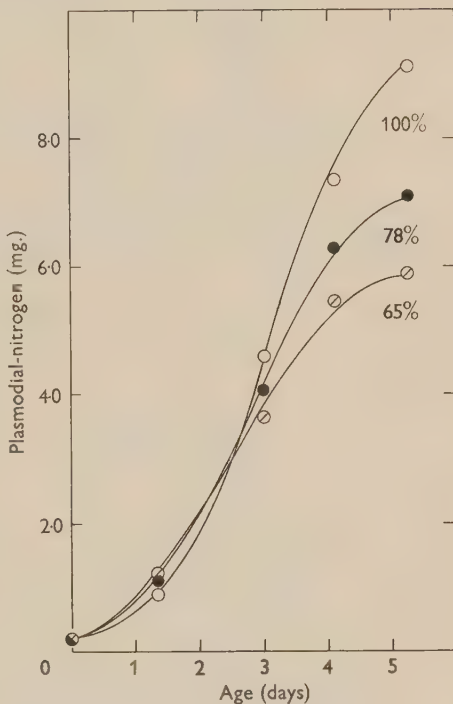


Fig. 6

Fig. 5. Ca⁺⁺ requirement for growth of *Physarum polycephalum*. Medium prepared as described in the text with the following exceptions: the phosphate buffer as described in Fig. 4 replaced the CaCO₃ buffer; CaCl₂·H₂O was omitted and added back to individual flasks to give indicated Ca⁺⁺ concentrations; 'added Ca⁺⁺' corrected for Ca⁺⁺ contained in inoculum. Inoculum: 1.0 ml. (equiv. 356 µg.-N)/culture, grown 4 days on above phosphate-buffered medium containing the CaCl₂·H₂O concentration indicated in Table 1. Cultures containing 20 ml. medium/500 ml. Erlenmeyer flask were harvested after incubation for 4 days at 21–22° and 170 recip./min. The yield for zero added Ca⁺⁺ was obtained from the third transfer of the culture on the phosphate-buffered medium with no added Ca⁺⁺. Plasmodial-N represents the average microKjeldahl-N of duplicate 21 ml. cultures.

Fig. 6. The effect of varying the culture volume on the plasmodial yield. Volume of medium/500 ml. Erlenmeyer flask: —○—, 100 ml.; —●—, 50 ml.; —○—, 20 ml. Cultures inoculated with 1.0 ml. (equiv. 200 µg.-N) of a 4-day culture (21 ml.)/20 ml. medium, incubated at 21–22° on an Eberbach reciprocal shaker having a 1½ in. stroke at 170 recip./min. Plasmodial-N represents the average microKjeldahl-N of duplicate 21 ml. cultures or calculated to a 21 ml. volume; % represents % of yield obtained with 20 ml. volume at 126 hr.

The amount of slime produced by the plasmodium also increased as indicated by increase in viscosity of the culture medium toward the end of the growth period, but no quantitative determinations were made.

Table 2. *Effect of inoculum size on growth rate*

Inoculum (<i>I</i>) ($\mu\text{g.}-\text{N}/20\text{ ml.}$)	Yield (<i>y</i>) ($\mu\text{g.}-\text{N}/20\text{ ml.}$)	Age (<i>a</i>) (hr.)	Duplication time (<i>t</i> _{av.}) (hr.)	Factor (<i>k</i>)
242	7100	114	25.8	1.3
450	7640	88	20.0	1.0
726	7040	80	23.8	1.2
1210	7160	69	28.3	1.4

I = inoculum, equiv. $\mu\text{g.}$ microKjeldahl-N in 20 ml. culture; 1.0 ml. = 450 $\mu\text{g.}-\text{N}$.

y = peak yield in $\mu\text{g.}$ microKjeldahl-N/20 ml. culture.

a = age of culture at peak yield.

*t*_{av.} = average plasmodial duplication time in hr. as defined by $t_{\text{av.}} = \frac{a}{3.3 \log y/I}$.

k = factor expressing the average time required for doubling of plasmodial growth relative to that required for a culture having an inoculum of equiv. 450 $\mu\text{g.}-\text{N}$.

Table 3. *Relation between duplication time and pigment formation*

Inoculum* (ml.)		Age of culture (hr.)				
		0	16	28	46	69
0.5	Duplication time (hr.)	14	16	18	22	—
	N/pig.	13.7	20.5	27.0	14.0	12.0
2.5	Duplication time (hr.)	19	19	20	—	—
	N/pig.	13.7	14.5	14.8	12.6	8.0

* ml. culture (equiv. 500 $\mu\text{g.}-\text{N}/\text{ml.}$) added to a submerged culture containing 20 ml. medium.

N/pig. = $\frac{\mu\text{g.}-\text{plasmodial-N}}{E_{415}}/20\text{ ml. culture.}$

Surface cultures

Plasmodia also grew very well on the surface of filter paper resting on glass beads at the surface of the nutrient medium. Under optimal conditions, a 1 ml. inoculum of a 3-day submerged culture, after fusing into a single plasmodium grew to a maximum diameter of 7 cm. in 3 days. Such plasmodia grew as thin compact circular disks. During maximal growth, the organism did not migrate but expanded peripherally; however, when the nutrients were exhausted or were replaced by a non-nutrient medium, the plaque-like culture quickly changed into a highly motile arborescent network. The growth of the plasmodium on the surface of nutrient agar was slower than that obtained on nutrient-moistened filter paper, and assumed a loose web-like appearance. Plasmodia floating on the surface of nutrient media also grew satisfactorily, but great care was required to prevent submersion of the organism.

DISCUSSION

The method of pure culture described in this paper has permitted the controlled production of *Physarum polycephalum* in sufficient quantity for critical studies on growth and morphogenesis. As far as the authors are aware, this report provides the first description of such growth conditions for a plasmodial myxomycete in axenic culture. The growth of *P. polycephalum* on a completely soluble medium refutes the conjecture often found in the literature that this plasmodial species requires particles or whole organisms for its nutrition.

The composition of the growth medium differs principally from that of media developed for other micro-organisms in that it contains embryo extract. Although a large number of soluble natural media were tested, the addition of embryo extract was always found necessary to obtain sustained growth. The required factor in embryo extract apparently occurs in few, if any, commercially processed soluble natural materials such as are usually tested for growth activity, and it was not present in various extracts of oats prepared in our laboratory, although rolled oats

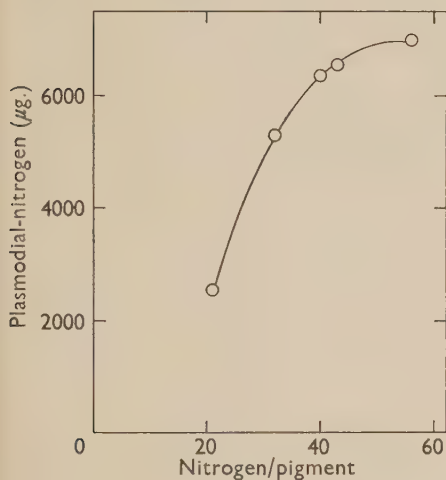


Fig. 7

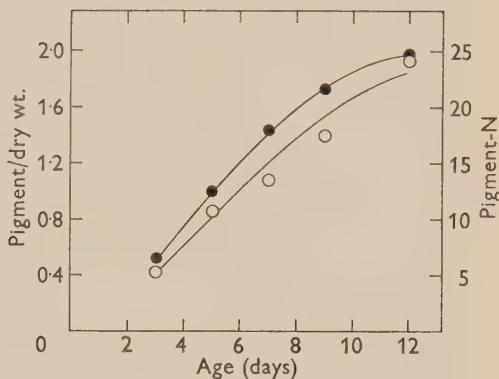


Fig. 8

Fig. 7. The relation between the nitrogen and pigment content of cultures grown on media of different Tryptone and glucose concentrations. Points represent nitrogen/pigment (N/pig.) for data shown in Fig. 2. Cultures containing 20 ml. medium and 1.0 ml. (equiv. 400 µg.-N) of 4-day inoculum were incubated for 4 days at 21–22° and agitated at 170 recip./min.

$$\frac{\text{Nitrogen}}{\text{Pigment}} = \left(\frac{N}{\text{pig.}} \right) = \frac{\mu\text{g. microKjeldahl-N}}{\text{total } E_{415}} \bigg/ 20 \text{ ml. culture.}$$

Fig. 8. The relation between the pigment content and the nitrogen and dry weight of plasmodia grown on oats. Triplicate oat cultures, 2 g./500 ml. Erlenmeyer flask, were each inoculated with a fragment from a similar 4-day oat culture, and incubated at 21–22° without agitation; 'oat-free' plasmodial samples were removed for analysis at the times indicated.

$$\text{—○—} = \frac{\text{Pigment } (E_{415})}{\text{Dry weight (mg.)}}; \quad \text{—●—} = \frac{\text{Pigment } (E_{415})}{\text{Nitrogen (N) } (\mu\text{g.})}.$$

Ratios represent average values for samples from triplicate cultures.

served as an excellent growth medium. The factor is readily extracted from chick embryo, several blood sera and erythrocytes. However, it cannot be replaced by the large number of pure metabolites so far tested. Embryo extract and blood serum have also been widely used for the growth of mammalian cells *in vitro* and, although the optimum concentration required for the growth of *P. polycephalum* is much lower, the resulting yield of organism is greater than for mammalian cells.

Although the maximum growth rate so far obtained with *Physarum polycephalum* on this medium is considerably less than that of many micro-organisms, the maximum yield is relatively high (equiv. 0.42 g. dry wt./g. glucose) and comparable to

the yields obtained with the aerobic filamentous fungi. However, *P. polycephalum* can also use Tryptone, to a small extent, as well as glucose, as an energy source. As the growth rate of ageing cultures declines, two readily detected products accumulate in the medium: a Sevag-precipitable viscous material, and the yellow plasmodial pigments. Estimation of the nitrogen content of these products is in progress to evaluate the efficiency of Tryptone for plasmodial growth as opposed to the formation of these by-products.

Physarum polycephalum, previously considered to grow only on surfaces as a large single plasmodium, can now be grown in submerged culture as a suspension of multinucleate microplasmodia. Such cultures facilitate the reproducible handling of this organism at any stage of growth under a variety of nutritional conditions, and expedite the preparation of uniform surface plasmodia in which synchronous mitoses can be most readily investigated. The small plasmodia from shaken cultures readily fuse under proper conditions within several hours, without appreciable deterioration, to form large (1–2 g.) plasmodia. This unique capacity of integrating rapidly and efficiently large numbers of small independent protoplasmic units into a single large stable plasmodium with little or no apparent loss of cellular material suggests the presence of a highly active and unusual cell interfacial boundary. Preliminary electron micrographs obtained by Dr I. B. Sachs in this laboratory confirm the presence of a very thin cell boundary and the absence of a grossly structured cell wall.

The mitotic synchrony first recognized by Howard (1932), and which occurs in plasmodia as large as 20 cm.² (Guttes *et al.* 1961) recommends *Physarum polycephalum* as an ideal material for studies requiring synchronous growth because: (a) synchronous mitosis is a natural and cyclic event (thus objections raised to the use of cell populations synchronized under conditions resulting in unbalanced cell multiplication or short-lived synchrony do not apply); (b) growth through a number of mitotic cycles can be studied in a single plasmodium or 'cell'. The advantage of such plasmodia for biochemical investigations was recently illustrated by the demonstration with conventional procedures that DNA is synthesized during a short period immediately following mitosis (Nygaard, Guttes & Rusch, 1960). Synchrony in this organism is probably dependent to a large extent on the rapid metabolic communication afforded by the rhythmic cytoplasmic flow among all areas of a single plasmodium.

Observations made during the present study indicate that migration of the plasmodium occurred only when the nutrient medium was suboptimal. Since protoplasmic streaming occurs in both migrating and non-migrating plasmodia, it appears that a decreased growth rate favours a directional net flow of the organism. The cause of streaming is unknown, but is thought to be associated with myxomyosin, a contractile protein (Kamiya, Nakajima & Abe, 1957; Ohta, 1954). This actomyosin-like protein was first detected by Loewy (1952) and further studied by Ts'o, Bonner, Eggman & Vinograd (1956).

The application of these present cultural methods also facilitates a more critical study of the life cycle. Vegetative plasmodia grown in agitated culture and fused into large surface plasmodia may be induced to sporulate under appropriate conditions of pure culture; the metabolic patterns which characterize this change are now being studied.

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The Survival of Stationary Phase *Aerobacter aerogenes* Stored in Aqueous Suspension

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SUMMARY

The survival characteristics of washed stationary phase *Aerobacter aerogenes* organisms suspended in buffered sodium chloride solution and stored at room temperature, or at 37° with aeration, depended on the medium used for growing the bacteria. Populations of bacteria harvested from tryptic meat broth or tryptone glucose medium remained almost completely viable for longer periods than bacteria from a simple ammonium salt + mannitol medium in which carbon was limiting. Analyses of washed freeze-dried preparations of freshly harvested bacteria showed that the amounts of protein, carbohydrate and ribonucleic acid present varied according to which of the above media was used for growth. During the initial stages of storage at 37°, when the viability of the population remained apparently unchanged, a progressive loss in bacterial dry weight occurred, due to degradation of these cell constituents. Endogenous glycogen was degraded and oxidized; bacteria which contained glycogen survived well. However, the addition of glucose to suspensions stored under aerobic or anaerobic conditions did not favour survival. Utilization of substances made available by degradation of various endogenous macromolecular constituents may be an important factor concerned with the survival of bacteria in unfavourable environments.

INTRODUCTION

Harrison (1960) showed that population density affected the survival of bacteria held under growth conditions in the absence of added nutrients. Products from dead or dying bacteria are utilized by the living organisms, allowing maintenance of viability or division to occur. It is possible that another important factor concerned with bacterial survival is the ability of a bacterium to maintain itself by utilizing some of its own internal constituents. The problem of energy reserve substances in bacteria was reviewed by Wilkinson (1959); he considered that these substances are probably homoglycans (glycogen), poly- β -hydroxybutyrate, other lipids (triglycerides) and polyphosphate. The accumulation and breakdown of internal glycogen was studied in *Escherichia coli* (Holme & Palmstierna, 1956*a-c*; Dagley & Dawes, 1949) and in yeast by Stier & Stannard (1935-36). Degradation of other macromolecular constituents occurs in bacteria maintained in the absence of added nutrients. For example, Holden (1958) reported that when *Lactobacillus arabinosus* was incubated in phosphate buffer at 37°, ribonucleic acid (RNA) was degraded, resulting in the release of ultraviolet (u.v.) absorbing substances into the

medium; a similar phenomenon was investigated in yeast (Higuchi & Uemura, 1959). Turnover of protein occurs in *Escherichia coli* maintained at growth temperature in the absence of nutrients (Mandelstam, 1958*a, b*; Mandelstam & Halvorson, 1960). We have studied the survival of stationary phase *Aerobacter aerogenes* organisms suspended in buffer solution and have investigated gross changes in the concentrations of endogenous RNA, protein and carbohydrate.

METHODS

The strain of *Aerobacter aerogenes* used was obtained from Professor Sir Cyril Hinshelwood's laboratory.

Media. (a) Defined medium usually contained (g./l.): NaH_2PO_4 , 0.6; $(\text{NH}_4)_2\text{HPO}_4$, 5.95; K_2SO_4 , 1.75; mannitol, 10; and salt mixture (5 ml.). Salt mixture was prepared by dissolving MgO (10 g.), CaCO_3 (1 g.), ZnO (0.16 g.), $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (2.7 g.) and $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (0.4 g.) in water (200 ml.) containing conc. HCl (50 ml.) and diluting to 1 l. with water. The final medium was sterilized by autoclaving (120° , 15 min.). (b) Tryptic meat broth, prepared from bullock's meat, contained 2.5 mg. nitrogen/ml. (c) Tryptone glucose (Wade, 1961). A concentrate was prepared by mixing three solutions (*A*, *B*, and *C*) together. *Soln. A*: 2.8 kg. of Tryptone (Oxo Ltd., London) were mixed with 1.05 l. of 2M- K_2HPO_4 and made up to 19.6 l. with water. The solution was adjusted to pH 9.3 with NaOH , brought to 100° for 5 min., then filtered (Green's filter-paper no. 904). Citric acid (112 g.) was added, the solution adjusted to pH 7.6 with HCl and the volume re-adjusted with water to 19.6 l. The solution was autoclaved in volumes of 2.8 l. at 115° for 30 min. *Soln. B*: 406 g. NaCl , 140 ml. m-ferric citrate, 14 ml. m- CaCl_2 and 560 ml. m- MgSO_4 were made up to 2.8 l. with water. Volumes of 750 ml. were autoclaved at 115° for 20 min. *Soln. C*: 2.8 kg. glucose were made up to 5.6 l. with water and the solution autoclaved in volumes of 1 l. at 115° for 10 min. The concentrate was prepared by mixing 2.8 l. of *soln. A*, 750 ml. of *soln. B* and 1 l. of *soln. C* together, and stored at $2-5^\circ$. The concentrate was diluted with sterile water (5 vol.), the final medium containing 1.8% (w/v) glucose.

Cultural conditions and harvesting. Organisms were grown at 37° in an apparatus (culture volume 0.5 or 3 l.) essentially as described by Elsworth, Meakin, Pirt & Capell (1956) and used as a batch culture vessel. The medium in the culture vessel was seeded with a suspension of organisms grown for 8 hr. at 37° in a flask containing the same medium, aerated by rocking on a shaker; the initial concentration in the culture vessel was about 10^8 living bacteria/ml. medium. Filtered air was passed through the impeller at 1 ml./min./ml. culture and the pH value was continuously maintained at 7.2. Cultures reached the stationary phase in 6 hr. or less with media described above. Bacteria were harvested by centrifugation, washed twice with buffer solution and resuspended at about 10^{10} bacteria/ml. in the same buffer solution. The solution used for washing and resuspending the organisms contained NaCl (0.13M) and $\text{KH}_2\text{PO}_4 + \text{K}_2\text{HPO}_4$ (0.02M- PO_4); final value pH 6.5.

Bacteria counting procedures. Total bacterial counts were made with a Thoma chamber and dark ground illumination (average of three determinations on each sample). Organisms within one-fifth of the ruled area were counted and samples were diluted with buffered saline (pH 6.5) so that the number present was 250-500. The

results obtained when 20 determinations were made on one sample varied between 298 and 368 (mean, 328; standard deviation, ± 21). Counts of viable bacteria were made by spreading 0.2 ml. sample (diluted by successive tenfold dilutions with buffered saline to contain 500–1000 viable organisms/ml.) on the dried surface of each of five nutrient agar plates. Colonies were counted after incubation for 24 hr. at 37°. When the dilution and plating procedure was repeated 20 times with one suspension, the number of colonies/ml. counted varied between 689 and 852 (mean, 777; standard deviation, ± 57). A direct determination of the % viable bacteria in a suspension was made by the slide culture method of Postgate, Crumpton & Hunter (1961) with tryptic meat broth agar and dark ground illumination. Microcolonies and undivided organisms were counted after incubation for about 3.5 hr. at 37°. When the % viability of one suspension was determined twelve times by this technique, results varied between 93.5 and 96.8 (mean, 95.7; standard deviation, ± 0.90).

Storage of suspensions of Aerobacter aerogenes. Sterility precautions were taken during the preparation, storage and sampling of bacterial suspensions. For storage at 37° with aeration, a suspension of washed freshly harvested organisms (usually 500 ml.; about 10^{10} bacteria/ml.; dry weight about 2.5 mg./ml.) at pH 6.5, was transferred to a sterilized gas wash bottle (1 l.; Dreschel pattern) held in a water bath at 37°. Washed filtered air was passed (0.2 l./min.) into the suspension through a sintered glass disk and the effluent air allowed to escape through a water reflux condenser fitted with a cottonwool filter. During the storage period, the pH value of the suspension was measured at intervals and, when necessary, adjusted to pH 6.5 by the slow addition of N-NaOH or N-HCl. Samples were removed at intervals for determinations of total and viable counts, % viability by the slide culture method, dry weights and chemical analyses. Samples for chemical analysis were centrifuged to separate the bacteria and the supernatant liquid was filtered through a Millipore bacteriological filter. The deposit of bacteria was washed with buffered saline by centrifugation and resuspended in water to known volume at a concentration of about 5 mg. dry weight/ml.; bacterial suspensions and filtered supernatant fluids were stored in the frozen state when not analysed immediately.

Analytical methods. Nucleic acids were determined in washed freeze-dried bacteria by the method of Schmidt & Thannhauser (1945); results were expressed in terms of nucleic acid phosphorus. Loss of RNA from *Aerobacter aerogenes* during storage was followed with the Schneider (1945) method applied to bacteria separated from suspensions by centrifugation; pentose in the hot trichloroacetic acid (TCA) fraction was estimated by the colorimetric method described by Morse & Carter (1949), or by the Dische & Borenfreund (1957) method, and converted to RNA by comparison of the colour obtained with that given by a known weight of yeast RNA (P, 8.1%; assumed to be 85% RNA). Bacterial pentose excluding pyrimidine pentose was determined with the Bial reaction (Morse & Carter, 1949) applied directly to a suspension; dried ribose (Laboratory Reagent Grade from The British Drug Houses Ltd., Poole) was used as the standard. Hexose interfered in the method and allowance was made for interfering carbohydrate in *A. aerogenes* by determining total carbohydrate in the bacterial suspension with the sulphonated resorcinol method of Devor, Conger & Gill (1958); dried ANALAR glucose from The British Drug Houses Ltd. was used as the standard. Light absorption was measured at 500 m μ

and, at this wavelength, ribose gave 96–102% of the colour given by an equal weight of glucose. In the Bial method, light absorption was measured at 660 m μ and glucose gave 11–15% of the reading given by an equal weight of ribose. If x = 'true glucose' ($\mu\text{g./ml.}$), y = 'true pentose excluding pyrimidine pentose' ($\mu\text{g./ml.}$), a = total carbohydrate found ($\mu\text{g./ml.}$) and b = pentose found ($\mu\text{g./ml.}$), then $x + 0.99y = a$ and $0.13x + y = b$; interference factors for x and y were determined for each batch of analyses. The results obtained for 'true pentose excluding pyrimidine pentose' by solving the equations, were close to values obtained for purine pentose in the hot TCA fraction (Schneider, 1945) and, in many experiments, RNA loss from suspensions was determined by application of the Bial and resorcinol methods. Total phosphorus in freeze-dried bacteria was determined by the method of King (1932) and total nitrogen by a Kjeldahl method after reduction of the sample with hydriodic acid and red phosphorus (Friedrich, 1933) in a Kjeldahl flask. Protein was determined by means of a Biuret method after the bacteria had been dissolved by heating in N-NaOH for 5 min. at 100° (Stickland, 1951); dried bovine plasma albumin (N, 15.9%) from Armour and Co. Ltd., Hampden Park, Eastbourne, was used as standard. Ammonia was determined by nesslerization after distillation of the sample in a Markham still with alkaline phosphate + borate buffer (Tracey, 1952). Keto acids were estimated by the method of Friedmann & Haugen (1943); individual keto acids were identified according to the paper chromatographic method of El Hawary & Thompson (1953). Sugars were identified by paper chromatography on Whatman no. 1 paper with ethyl acetate + pyridine + water (2 + 1 + 2, v/v) as solvent (Jermyn & Isherwood, 1949) and aniline phthalate as spray reagent (Partridge, 1949). For the paper chromatographic detection of amino acids, butanol + acetic acid + water (4 + 1 + 5, v/v) was used as solvent and ninhydrin as the spray reagent. Free amino acids were extracted from bacteria by heating suspensions in distilled water (20 mg. dry wt./ml.) for 20 min. The extract was separated by centrifugation and desalted by passage through a column of Amberlite IR-120 (H form). The column was washed with water and the amino acids eluted with pyridine water (10%, v/v). The eluate was concentrated to small volume and chromatographed. Free amino acids released into the suspending medium during storage were detected after the suspension was freed from organisms by centrifugation and filtration through a Millipore filter. The filtrate was desalted in an electrolytic desalting apparatus, reduced to a small volume and chromatographed. Ultraviolet absorption was measured in a Unicam quartz spectrophotometer, model S.P. 500, with a 1 cm. light path. The dry weight of a suspension was determined by centrifuging a measured volume, washing the deposit once with saline (NaCl, 0.85%, w/v) containing HCHO (3%, w/v) and once with water, and drying the deposit at 100° for 16 hr.

Isolation of glycogen from Aerobacter aerogenes harvested from tryptone glucose medium. Freeze-dried organisms (1.5 g.) suspended in water (50 ml.) were disintegrated in the presence of ballotini beads (size 12) and capryl alcohol in a Mickle (1948) tissue disintegrator. The homogenate was centrifuged at 6000 g for 30 min. and the supernatant liquid separated. TCA solution (25%, w/v) was added to the cold supernatant liquid to a concentration of 2.5% (w/v) TCA and the precipitate separated by centrifugation. The supernatant liquid was neutralized by addition of solid NaHCO_3 and dialysed against running distilled water at 2° for 24 hr. The sac

contents were concentrated to 16 ml. under reduced pressure and treated with 3 volumes of ethanol and 2–3 drops saturated ethanolic potassium acetate solution. After standing for 24 hr. at 2° the precipitate was isolated by centrifugation, washed with ethanol and dried *in vacuo*. The yield was 140 mg. (= 9.3 % dry wt. bacteria); carbohydrate content, 85 % (as glucose). Further fractionation with ethanol resulted in a fraction containing 97 % carbohydrate (as glucose) which, after acid hydrolysis and paper chromatography, gave one spot corresponding in position to glucose. The isolated material gave an opalescent solution in water and reacted with iodine solution to give a reddish violet colour.

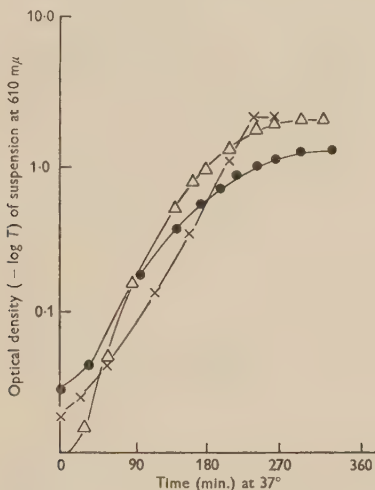


Fig. 1. Growth curves for *Aerobacter aerogenes* in defined medium (x), tryptic meat broth (●) and tryptone glucose medium (Δ).

RESULTS

Growth curves for *Aerobacter aerogenes* grown in defined medium, tryptic meat broth and tryptone glucose medium in the fermenter are shown in Fig. 1. Growth was determined by turbidity measurements at 610 mμ in a Coleman Junior spectrophotometer with the appropriate medium as blank and, when necessary, the sample and blank were diluted with buffered saline (pH 6.5) so that the optical density reading was not greater than 0.25. The shape of the growth curve depended on the medium used and with the defined medium the change from the exponential phase into the stationary phase was sharp whereas, with either of the complex media, the turn was more gradual. Bacteria were harvested after growth had ceased for 0.5 hr. unless otherwise stated. The viability of washed suspensions of bacteria freshly harvested from any of the three media was high (93–99 %) according to the slide-culture method of Postgate *et al.* (1961).

The Postgate *et al.* method determines only the % viability of a suspension and not the total number of bacteria present. It was important in the present work, to know whether the total number of organisms in a given suspension changed during storage and, for this reason, total and viable counts were determined. In our hands,

the coefficients of variation for the results of slide-culture viability determinations, total counts and viable counts were ± 0.95 , ± 6.4 and $\pm 7.4\%$ respectively (see Methods). Evidently results for the % viability of suspensions determined by the slide-culture method were more reliable than those derived from a combination of total and viable counts, provided the generation time of individual organisms did not exceed the incubation period allowed (3.5 hr.). A comparison of the results for % viability of an aerated suspension of *Aerobacter aerogenes* during storage at 37° as determined by the slide-culture technique and from viable and total counts is shown in Table 1. The values obtained with the Postgate *et al.* method were usually higher than those derived from the other methods. On the other hand, when the % viabilities of more aged suspensions were determined, it was frequently found that values by the slide culture method were lower than those derived from total and viable counts. This was probably related to the long lag phase of a proportion of an aged population. The incubation period of slide cultures could not be extended much beyond 3.5 hr. otherwise large colonies from single organisms with short generation times overgrew the non-growing bacteria. The shapes of survival curves plotted with data obtained by either the slide-culture technique or by total and viable counts were similar for periods during which biochemical changes occurring within the bacteria were studied.

Table 1. Comparison of the results obtained for the % viability of an aerated suspension of stationary phase *Aerobacter aerogenes* during storage at 37° determined (a) by the method of Postgate *et al.* (1960); (b) from total and viable counts

Organisms harvested from tryptic meat broth were washed twice with sodium chloride + potassium phosphate solution (pH 6.5) and resuspended in the same solution.									
Time of storage (hr.) ...	0	16	24	40	48	64	72	88	
(1) Total cell count $\times 10^{-10}$	1.13	1.11	1.27	1.16	—	—	—	1.17	
(2) Viable cell count $\times 10^{-10}$	0.98	0.95	0.90	0.96	0.95	0.75	0.73	0.60	
(3) % viability $((2)/(1) \times 100)$	87	86.5	71	83	—	—	—	51	
(4) % viability by the method of Postgate <i>et al.</i> (1961)	97.5	98	96	90	87	78	68	59	

Some of the factors which affected survival of washed bacteria were investigated. In the pH range 4–9 with Michaelis (1931) constant ionic strength buffers, the optimum value for survival of suspensions (about 10^{10} organisms/ml.) stored at 20 – 22° under nitrogen was near pH 6.5; in the range pH 5–8 with orthophosphate buffers (0.02 M PO_4) in sodium chloride (0.13 M) solutions, a similar pH optimum was found with suspensions stored under these conditions or at 37° with aeration. A solution of buffered sodium chloride (pH 6.5) was used routinely for washing and suspending bacteria. This solution appeared to be non-toxic since bacterial suspensions stored at 20 – 22° under air or nitrogen survived almost completely for days and in some cases for weeks. It was found that at 37° with aeration, the initial bacterial concentration affected survival of a population. Initial concentrations of less than 10^7 organisms/ml. multiplied to 10^6 – 10^7 /ml. within 70 hr. This is in agreement with the work of Garvie (1955) who found that *Escherichia coli* multiplied in buffer solution prepared from specially purified salts dissolved in distilled water. Above a

concentration of 10^7 – 10^8 bacteria/ml. significant multiplication did not occur and the length of the initial period of almost complete survival decreased as the concentration of organisms was increased to 10^{10} /ml. A concentration of about 10^{10} organisms/ml. was used routinely because this avoided maintenance of a significant proportion of the population by contaminating nutrients present in the buffer and also was convenient for subsequent chemical analyses. The shape of survival curves for suspensions of bacteria, particularly those harvested from defined medium, stored at a concentration of about 10^{10} /ml. sometimes showed evidence of regrowth; this was probably due to the utilization of material obtained from dying or dead organisms (Harrison, 1960). Regrowth was decreased when bacterial suspensions in cellophan sacs were dialysed against buffered saline. The survival of bacteria depended on the growth phase of the organisms at the time of harvesting. Survival curves for bacteria harvested from defined medium during the late exponential growth phase and at different times in the stationary phase (Fig. 2) showed that extension of the stationary phase under growth conditions favoured survival. The composition of the medium used for growing the bacteria affected their subsequent survival in buffered saline. Organisms harvested from tryptone glucose medium survived best and those harvested from tryptic meat broth usually survived better than bacteria grown in carbon-limiting defined medium. This was true during storage at 37° with aeration (Fig. 3), or at 20 – 22° under nitrogen; under the latter conditions the initial periods of almost complete survival were about 15, 10 and 4 days for organisms harvested from tryptone glucose medium, tryptic meat broth and carbon-limiting defined medium, respectively.

Analyses of bacteria grown in different media. Analyses of well-washed freeze-dried stationary phase bacteria harvested from the three media are shown in Table 2. On a dry-weight basis, bacteria grown in tryptone glucose medium contained 3–4 times as much carbohydrate (as glucose) excluding pentose and about half as much RNA as bacteria grown in the defined medium. Bacteria grown in tryptic meat broth contained a relatively large amount of protein and less RNA than did bacteria from the defined medium. Poly- β -hydroxybutyric acid (Lemoigne, 1927; Forsyth, Hayward & Roberts, 1958) was not detected in chloroform extracts of 1 g. amounts of freeze-dried bacteria which had been grown in any of these media. Besides differences in chemical analyses the bacteria varied in average weight according to whether they were grown in defined or complex medium (Table 2). Stationary phase bacteria harvested from defined medium were metabolically more active than bacteria from tryptone glucose medium. For example, Q_{O_2} (μ l./hr./bacterium) values for washed bacteria during the period when the rate of oxygen uptake was constant, were 5.2 and 2.36×10^{-9} for organisms from the defined medium and from the tryptone glucose medium, respectively; in the presence of mannitol (1%, w/v) Q_{O_2} values of 2.07 and 0.64×10^{-7} , respectively, were obtained.

The high carbohydrate content of bacteria harvested from tryptone glucose medium (Table 2) was due to the presence of glycogen; this was isolated from the organisms (see Methods). Significant amounts of glycogen were not found in homogenates of organisms harvested from carbon-limiting defined medium or from tryptic meat broth.

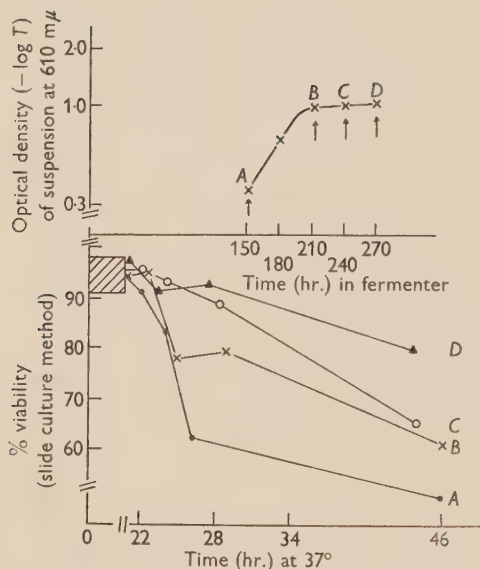


Fig. 2

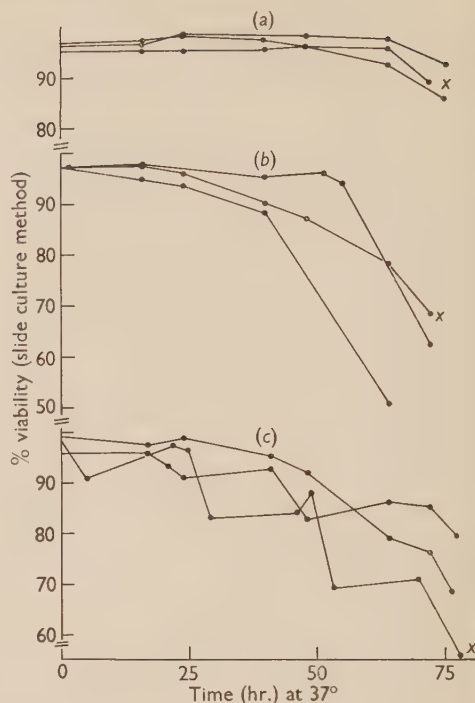


Fig. 3

Fig. 2. Survival of *Aerobacter aerogenes* harvested from defined medium during the exponential and stationary phases of growth. Arrows on upper curve indicate when samples (A-D) were taken. Lower curves show survival of washed bacteria from samples A-D during storage in buffered saline (pH 6.5) at 37° with aeration. Viability determined by the method of Postgate *et al.* (1961). Viability of all four suspensions determined at 0, 1, 2, 3, 4, 5 and 20 hr. was 91-98%; this is indicated by the hatched area.

Fig. 3. Survival curves for *Aerobacter aerogenes* harvested 0.5 hr. after cultures had reached stationary phase in: (a) tryptone glucose medium; (b) tryptic meat broth; (c) defined medium. Washed bacteria were stored in buffered saline (pH 6.5) at 37° with aeration. Curves marked 'x' and Figs. 4-9 refer to the same experiments (see text).

Table 2. Analysis of washed freeze-dried stationary phase *Aerobacter aerogenes* harvested from different media

Medium	Average dry wt./ bacterium (g. × 10 ¹³)	Total carbohydrate					RNA P	DNA P
		Total N	Total P	Total protein	Total excluding pentose	Total pentose*		
(g./100 g.)								
Defined	3.47	13.6	2.77	66	5.8	5.6	1.66	0.31
Tryptone glucose	2.42	11.6	1.95	60	15-20	3.7	0.87	0.38
Tryptic meat broth	2.25	13.9	2.14	72	3.8	4.0	1.09	0.49

* Excluding pyrimidine pentose

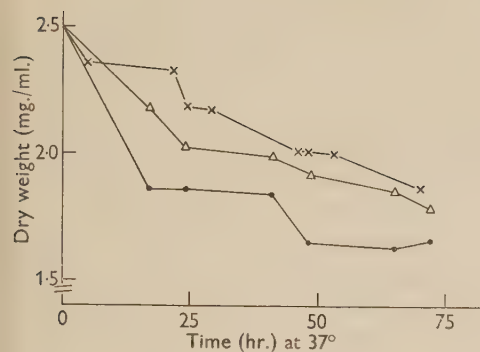


Fig. 4. Loss of dry weight.

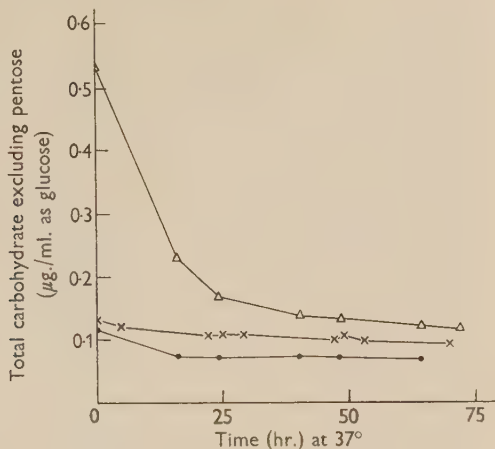


Fig. 5. Loss of glycogen.

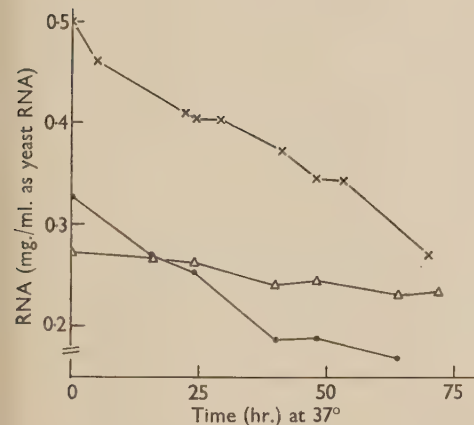


Fig. 6. Loss of RNA (determined by the Schneider, 1945, method).

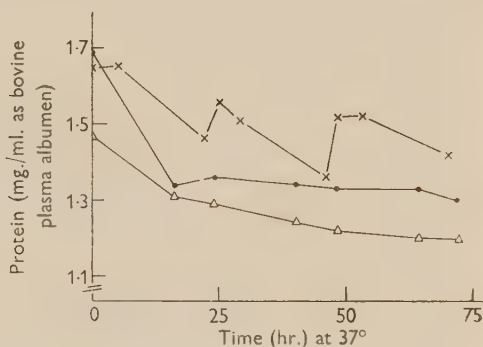


Fig. 7. Loss of protein.

Figs. 4-7. Loss of macromolecular constituents from washed *Aerobacter aerogenes* during storage in buffered saline (pH 6.5) at 37° with aeration. Bacteria harvested after 0.5 hr. in stationary phase from tryptone glucose medium (Δ), tryptic meat broth (●) and defined medium (×).

Losses of protein, RNA and glycogen from stationary phase bacterial suspensions

Suspensions of washed bacteria (about 10^{10} /ml.) freshly harvested from the three media were incubated at 37°, with aeration, in gas wash bottles. Samples were removed at intervals for determinations of pH value, total bacterial count, viability and chemical analysis. The data shown (Figs. 4-9) are those for one typical experiment with bacteria harvested from each of the three media; in each case the results were adjusted for a dry weight of 2.5 mg. organisms/ml. at $t = 0$. Survival curves referring to the suspensions investigated are shown in Fig. 3*a-c* (×). Considering first bacteria harvested from tryptone glucose medium, their initial viability of 95% was maintained for at least 65 hr. (slide-culture method; Fig. 3*a*, ×). At 0, 16, 24, 48 and 64 hr., the total counts were 1.23, 1.22, 1.20, 1.30 and 1.18 and viable counts

0.94, 0.98, 0.97, 0.93 and 0.96×10^{10} organisms/ml., respectively. Thus the substantial loss of dry weight which occurred in 65 hr. (about 25 %; Fig. 4) apparently represented a loss from living bacteria. The dry-weight loss was due mainly to losses of glycogen (0.41 mg./ml.; Fig. 5), RNA (0.04 mg./ml.; Fig. 6) and protein (0.27 mg./ml.; Fig. 7). Endogenous glycogen appeared to be metabolized by the bacteria because (1) the carbohydrate content of the supernatant fluid from the suspension did not increase significantly during storage; (2) the keto acid content of the organisms increased about fourfold during the first few hours of storage and then decreased to near the original value in 17 hr. The keto acid increase was due mainly to the formation of pyruvic acid. During the period when endogenous keto acid concentration increased, the rate of glycogen loss was high (Fig. 5). The suspension of bacteria harvested from tryptic meat broth had an initial viability of 97.5 % and this was maintained for about 40 hr. (slide-culture method; Fig. 3*b*, \times); the respective total and viable counts for this suspension are shown in Table 1. Again there was a loss of dry weight during storage (about 25 % in 40 hr.; Fig. 4) mainly due to losses of RNA (0.14 mg./ml.; Fig. 6), protein (0.35 mg./ml.; Fig. 7), and total carbohydrate excluding pentose (0.04 mg./ml.; Fig. 5). Survival curves for bacteria harvested 0.5 hr. after growth had stopped in carbon-limiting defined medium were usually less regular than those for organisms from the complex media (Fig. 3*c*); the initial viability of the suspension discussed here was 96 %, and this was more or less maintained for 40 hr. (Fig. 3*c*, \times). At 0, 17, 24, 41 and 64 hr., total counts were 0.70, 0.80, 0.70, 0.73, 0.74 and viable counts 0.59, 0.54, 0.68, 0.59, 0.40×10^{10} organisms/ml., respectively. The loss of dry weight (about 20 %, Fig. 4) which occurred in 40 hr. was mainly due to losses of RNA (0.15 mg./ml.; Fig. 6), protein (0.17 mg./ml.; Fig. 7) and total carbohydrate excluding pentose (0.03 mg./ml.; Fig. 5). In further experiments, the disappearance of RNA from these bacteria was shown to be most rapid during the first 8 hr. (average loss, about 2.0 %/hr.) and the rate decreased during the next 16 hr. (average loss, about 1.0 %/hr.).

In parallel with the losses of endogenous constituents there was an excretion into the surrounding medium of material which included ammonia (Fig. 8) u.v.-absorbing substances (Fig. 9) and traces of free amino acids. Bacteria harvested from tryptic meat broth which initially contained the largest amount of protein (68–72 % dry weight), excreted the largest amount of ammonia. Since the total nitrogen of RNA lost from this suspension during storage for 40 hr. was equivalent to about 22 $\mu\text{g./ml.}$ and the ammonia-N excreted was 65 $\mu\text{g./ml.}$ it seems probable that some of the ammonia was released as a result of protein degradation. During storage, the concentration of free amino acids in the bacteria or in the surrounding medium did not change sufficiently to explain the protein loss as being due to proteolysis alone. Bacteria harvested from defined medium, from which a considerable amount of RNA was lost (Fig. 6), excreted the largest amount of u.v.-absorbing material; some of this was re-absorbed, presumably by living organisms (Fig. 9). The absorption spectrum of the excreted material showed a broad flat peak extending from 248 to 257 $m\mu$, similar to that of the material excreted by *Lactobacillus arabinosus* and investigated by Holden (1958). Analysis of this material by ion-exchange resin chromatography and paper ionophoresis, kindly done by our colleague Mr H. E. Wade, showed that the u.v.-absorbing substances were mainly free bases with a relatively high proportion of hypoxanthine. The presence of hypoxanthine suggested

that, during storage, adenine nucleotides were degraded, resulting in the release of ammonia and ribose. Since the concentration of pentose, as well as endogenous RNA, decreased, it is evident that ribose was metabolized. Similar losses of RNA, glycogen and protein occurred when bacterial suspensions were held at 20–22° under nitrogen, but at a slower rate.

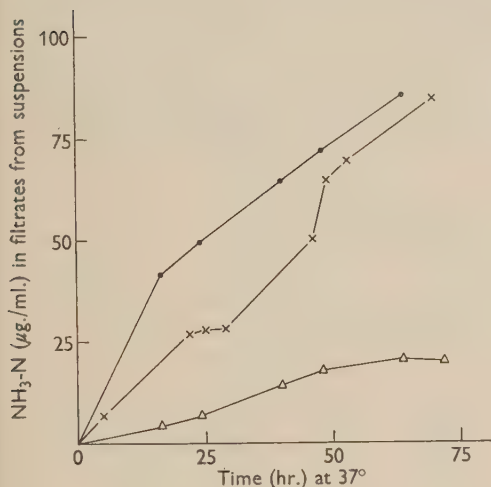


Fig. 8

Fig. 8. Excretion of ammonia by stationary phase *Aerobacter aerogenes*. Growth media, conditions of storage and symbols as for Fig. 4.

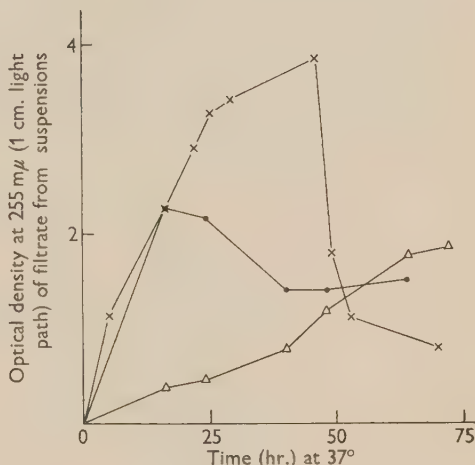


Fig. 9

Fig. 9. Excretion of u.v.-absorbing substances by stationary phase *Aerobacter aerogenes*. Growth media, conditions of storage and symbols as for Fig. 4.

Synthesis of endogenous glycogen by bacteria grown in defined medium

The defined medium described in Methods contained 1% (w/v) mannitol and 0.12% (w/v) nitrogen as ammonium salt. Growth was limited by the mannitol and when this had been utilized about 55% of the nitrogen had been used. When the ammonium salt concentration was decreased by 75%, nitrogen became limiting and, as reported by Holme (1957), glycogen was laid down in the organisms during the stationary phase. The results of a typical experiment are shown in Fig. 10. Bacteria were harvested after 4 hr. in the stationary phase from both nitrogen-limiting medium and the normal carbon-limiting medium, and their survival characteristics were compared. The glycogen-containing bacteria survived better than did those without reserves of glycogen.

Effect of added glucose on the survival of bacteria in buffered saline

Stationary phase bacteria freshly harvested from tryptic meat broth were washed by centrifugation and suspended at a concentration of about 0.6×10^{10} viable organisms/ml. in buffered saline (pH 6.5). Glucose (to 0.45%, w/v) was added and the suspension incubated at 37° with aeration. During incubation the pH value of

the suspension was controlled by the addition of NaOH. During a 4 hr. incubation period, samples were removed for viability determinations and chemical analyses. It was found that about 5% of the added glucose was turned into cellular glycogen and about 60% was metabolized (Fig. 11). During the incubation period, 50% of the bacteria became non-viable; apparently this was due to the presence of glucose, for similar suspensions without added sugar remained completely viable during the experimental period. When similar suspensions containing added glucose were incubated at 37° under anaerobic conditions (nitrogen) the Pasteur effect was evident, i.e. the substrate was fermented much more rapidly. No cellular glycogen was formed under these conditions. Again about 50% of the population died during a 4 hr. incubation period.

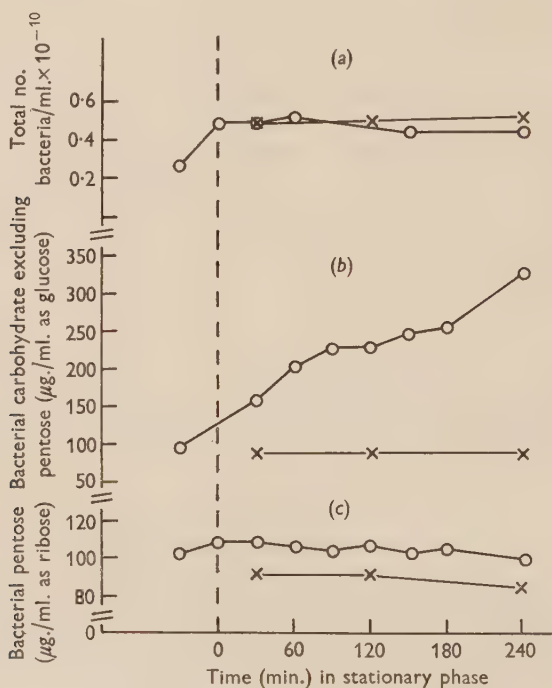


Fig. 10. Synthesis of endogenous glycogen by *Aerobacter aerogenes* grown in defined medium. Bacteria grown in nitrogen-limited medium (O); (a) total count, (b) total carbohydrate excluding pentose in washed cells, (c) total pentose in washed organisms. Some results for bacteria grown in carbon-limited defined medium are shown for comparison (X).

DISCUSSION

In the present work, populations of stationary phase organisms of *Aerobacter aerogenes* were grown in a culture vessel under controlled conditions and, in a given medium, their survival characteristics, mean mass and amounts of protein, ribonucleic acid and carbohydrate did not vary greatly from batch to batch. Suspensions of bacteria grown in this way had high initial viabilities which were maintained for periods of hours or days, according to the medium used for growth and the conditions of storage. During this period, degradative changes occurred within the bacteria resulting in the loss of up to 25% of dry weight. Products made available

by these degradative processes were metabolized by the living organisms and it appeared that their viability was maintained as a result of this. Once a proportion of a bacterial population dies, a comparatively large amount of nutrient material from the corpses may eventually become available to the survivors, thus allowing some of them to grow and divide. When division occurs the population is no longer the original one; in any case chemical changes found to occur after this are difficult

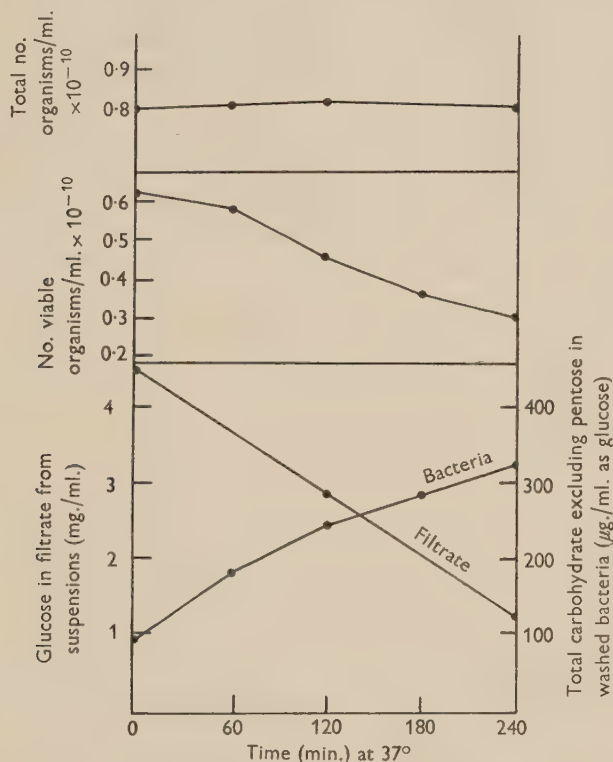


Fig. 11. The effect of exogenous glucose on the survival of *Aerobacter aerogenes* suspensions stored at 37° with aeration. Bacteria harvested from tryptic meat broth were washed and resuspended in buffered saline (pH 6.5). Glucose (4.5 mg./ml.) was added at zero time.

to interpret since they are occurring in viable and/or dead bacteria. The phenomenon of re-growth in suspensions of *A. aerogenes* held at growth temperature in the absence of nutrients was studied by Harrison (1960). It may be prevented to some extent by dialysing suspensions in cellophan sacs; with such suspensions the survival curve usually showed an initial period of almost complete survival followed by a period of nearly exponential death. This appears to substantiate the hypothesis that bacteria may survive in an unfavourable environment at the expense of endogenous constituents to the stage when further utilization affects cell integrity.

Results of previous studies concerned with the role of glycogen in bacteria and yeasts suggest that in these organisms the substance does not always fulfil the criteria of an energy reserve as it does in animals (Holme, 1957). For example,

Stier & Stannard (1935-36) showed that the glycogen of yeast cells decreased slowly when they were suspended in a carbon-free medium, but it was not fermented. Holme (1957) reported that utilization of internal glycogen by *Escherichia coli* occurred during rapid growth in a complete medium. In bacteria starved of an organic carbon source but supplied with nitrogen, no increase in nitrogen-containing compounds occurred in spite of the fact that 6-8 % of the dry weight of the organisms was glycogen. The fact that bacteria lose internal glycogen during growth and division does not necessarily affect the hypothesis that glycogen serves as a reserve material for stationary phase organisms in an unfavourable environment. Also the fact that bacteria containing 6-8 % glycogen, when put into nitrogen-containing medium, did not synthesize nitrogen-containing compounds is perhaps not so important as whether or not the organisms survived. On this aspect no data are given by Holme and his colleagues. Holme & Palmstierna (1956*d*) reported other experiments in which the utilization of glycogen-carbon for synthetic purposes was shown and, as pointed out by Wilkinson (1959), in these experiments internal glycogen did fulfil the criteria of an energy-reserve substance. The results of our survival studies showed that stationary phase organisms containing glycogen survived well in an unfavourable environment. It was found that when cell-free extracts of mechanically disrupted *Aerobacter aerogenes* were incubated at 37° with purified glycogen (see Methods) and buffer (pH 7.2), glucose was released. No free glucose was detected in control tubes containing glycogen without extract or extract without glycogen, incubated in parallel. Thus *A. aerogenes* had the mechanism for degrading the polysaccharide and the energy derived from glucose metabolism may have contributed towards the better survival characteristics of glycogen-containing bacteria. However, when glucose was added to an aerated suspension of organisms in buffered saline at 37°, endogenous glycogen equivalent to about 5 % of the added sugar was formed in 4 hr. and most of the remaining glucose was metabolized. During the incubation period about 50 % of the population lost their viability. It is possible that the metabolism of a relatively large amount of glucose in the absence of a nitrogen source caused the degradation or denaturation of internal substances, such as enzymes, which could not be replaced.

It has been shown that the ribonucleic acid of stationary phase *Lactobacillus arabinosus* stored in buffer at 37° is degraded (Holden, 1958); in our experiments with *Aerobacter aerogenes* a similar process occurred which had no immediate effect on viability. Stephenson & Trim (1938) showed that washed suspensions of *Escherichia coli* were able to degrade adenine nucleotides and that the products of the reaction were hypoxanthine, inorganic phosphate, ammonia and ribose; ribose did not accumulate but was fermented. Eggleston & Krebs (1959) extended this work. We found that when cell-free extracts of *A. aerogenes* were incubated with adenosine at 37° and pH 8.4, ammonia and ribose were released and the peak of the reaction mixture absorption spectrum minus the absorption spectrum of the extract shifted from 257 to 247 m μ , the latter being the wavelength of maximum absorption of hypoxanthine. Similarly cytidine was converted to uracil on incubation with cell extract. The excretion of free bases, including hypoxanthine, by suspensions of viable *A. aerogenes* suggests that the degradation of ribonucleic acid may provide the bacteria with readily metabolizable sources of carbon (ribose) and nitrogen (ammonia).

Data about protein turnover in *Escherichia coli* maintained at growth temperature in the absence of nutrients are available from the reports of Mandelstam (1958*b*) and Mandelstam & Halvorson (1960). The rate of protein-degradation was equal to the rate of protein synthesis at about 5%/hr. It was suggested that the pool of amino acids made available by this process was used for the production of inducible enzymes to cope with changed chemical environments (Mandelstam, 1956; Pollock, 1958). The data were obtained for suspensions incubated for up to 4 hr. The results of the present work show that in an unfavourable environment for longer periods, degradation of protein in *Aerobacter aerogenes* was not in equilibrium with synthesis and apparently went beyond the amino acid stage, resulting in the release of ammonia. This loss of protein did not immediately affect viability, and in some cases up to 25% of the total cell protein was lost before significant deaths occurred in the population. It is evident that the concentrations of major constituents such as protein, ribonucleic acid and glycogen in bacteria vary according to the medium used for their growth and that these substances may be present in excess of the amounts needed for immediate use. It is difficult to prove unequivocally that utilization of these substances allows cells to survive, but the shape of the survival curve obtained in the present work, showing an initial lag period before the population started to die, could be explained on this basis.

We are indebted to Dr D. Herbert for much useful advice and discussion.

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Analysis of a Syntrophic Growth of *Lactobacillus plantarum* and *Streptococcus faecalis*

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SUMMARY

Syntrophism was demonstrated between strains of *Lactobacillus plantarum* and *Streptococcus faecalis* in a chemically defined medium which lacked phenylalanine and pteroylglutamic acid (PGA); however, the presence of *p*-aminobenzoic acid (PABA) was essential. Enumeration of the two organisms in syntrophic growth was found to be possible by the use of Trypticase soy agar + 0.25% (w/v) glucose at pH 9.0 as a selective medium for *S. faecalis*. It was found that *S. faecalis* predominated in the early stages of the syntrophic growth, but that *L. plantarum* eventually predominated. Characterization by a bioautographic technique of the phenylalanine-active factor synthesized by *S. faecalis* and required by *L. plantarum* indicated that the factor was not identical with phenylalanine or shikimic acid. Microbiological assays of the culture fluid from cultures of *L. plantarum* indicated that three types of folic acid compounds were synthesized: (1) oxygen-stable compounds which supported the growth of *Pediococcus cerevisiae*; (2) oxygen-labile compounds which supported the growth of *P. cerevisiae*; (3) oxygen-stable compounds which supported the growth of *S. faecalis* but not of *P. cerevisiae*.

INTRODUCTION

Syntrophic growth (mutual feeding) of *Lactobacillus plantarum* strain 17-5 with *Streptococcus faecalis* strain R was first reported by Nurmikko (1954). In a chemically defined medium which lacked phenylalanine (required by *L. plantarum*) and pteroylglutamic acid (PGA; required by *S. faecalis*), neither organism grew alone; but in mixed culture, abundant growth of both organisms occurred. It was suggested that this syntrophic growth occurred because each organism synthesized the omitted growth factor required by the other, i.e. phenylalanine was synthesized by *S. faecalis* and PGA was synthesized by *L. plantarum*. Koft & Morrison (1956) reported much the same phenomenon. Judge (1958) studied this and other examples of syntrophism, and analysed the culture filtrates of *S. faecalis* to determine the nature of the phenylalanine-active substance synthesized and present in the culture filtrate. A one-dimensional ascending chromatographic technique was used, and the presence of a spot, corresponding to the position of phenylalanine, was demonstrated with a chemical indicator.

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The purpose of the present investigation was directed toward a further analysis of this system of syntrophic growth, and can be divided into two parts: (1) population aspects of the syntrophism, (2) the nature of the factor(s) synthesized by each of the two organisms involved in the mutual growth promotion.

METHODS

Organisms. The two organisms used in the syntrophic growth experiments were *Lactobacillus plantarum* strain 17-5 (American Type Culture Collection, Washington 7, D.C., U.S.A., ATCC No. 8014) and *Streptococcus faecalis* strain R (ATCC No. 8043). For the microbiological assay of folic acid-like compounds, the following organisms were used, in addition to *S. faecalis*, namely, *Lactobacillus casei* (ATCC No. 7469) and *Pediococcus cerevisiae* (ATCC No. 8081). All cultures were maintained in slabs of Micro assay culture agar (BBL; Baltimore Biological Laboratory, Inc., Baltimore 18, Maryland, U.S.A.) in the refrigerator: new transfers were made every 2-3 weeks.

Media. The defined medium used was that described by Nurmikko (1954) except for variations in the phenylalanine, *p*-amino benzoic acid (PABA) and PGA content. The composition of this medium is also described in an earlier report from this laboratory (Krieg & Pelczar, 1960). The term basal medium, as used here, refers to Nurmikko's medium minus *p*-aminobenzoic acid (PABA), pteroylglutamic acid (PGA) and phenylalanine.

Analysis of syntrophic growth. *Lactobacillus plantarum* and *Streptococcus faecalis* were grown separately in the basal defined medium supplemented with 200 μ g. phenylalanine, 0.2 μ g. PABA and 0.01 μ g. PGA/ml. After incubation for 18 hr. the cultures were centrifuged, washed twice with sterile physiological saline, and then resuspended in sterile distilled water to an optical density of 0.150 at 420 m μ as measured in a 16 mm. cuvette with a Bausch and Lomb Spectronic 20 colorimeter. These suspensions were then further diluted 1/10 in sterile distilled water. These dilutions were used as inocula (0.1 ml./tube containing 5.0 ml. medium) and, respectively, contained about 6.5×10^6 viable organisms of *S. faecalis*, or 7.5×10^6 of *L. plantarum*/ml. The scheme of inoculation (single cultures and cultures in admixture) to assess the effect of PGA, PABA and phenylalanine is evident from Fig. 1. It can be seen that the variations in media concerned the phenylalanine, PABA, and PGA content, and variations in inocula concerned the use of single cultures of each organism or both organisms inoculated together into the same medium. All incubations were at 35°, for various times (Fig. 1) in an air incubator. Growth was measured turbidimetrically at 600 m μ in 16 mm. optically matched screw-capped tubes.

In experiments where the two syntrophic organisms were enumerated separately from the mixed culture (Table 1), the inoculum was prepared as described above except that the concentrations of PABA and PGA were changed to 0.002 and 0.05 μ g./ml., respectively. Growth was measured by three methods: direct count, colony count, turbidimetrically (Table 2).

Analysis of Lactobacillus plantarum culture medium for folic acid activity. For the production of the folic acid-like factor(s) synthesized by *Lactobacillus plantarum*, 5.0 ml. basal medium supplemented with 200 μ g. phenylalanine and 0.05 μ g. PABA/ml. were inoculated from the stock stab culture. After incubation for 24 hr.,

a second transfer was made to the same medium. After a further incubation for 18 hr., 1.0 ml. of the culture was added to 300 ml. of the same medium supplemented with an excess of PABA (1 mg./ml.). After incubation for 24 hr. the culture was divided into two portions: one portion was untreated, while ascorbic acid (10 mg./ml.) was added to the other. Both portions were centrifuged to remove the organisms and the supernatant fluids adjusted to pH 6.7 with a measured quantity of 2N-NaOH. The two portions of supernatant fluid were kept frozen at -10° until assayed for folic acid activity; the portion which contained ascorbic acid was held in an atmosphere of argon.

Assays with *Pediococcus cerevisiae* were made with the medium of Sauberlich (1949); 6.0 ml. of assay medium were used per tube. When assays were performed with the ascorbic acid-containing portion, 1.33 mg. ascorbic acid was added to each ml. of single-strength assay medium, which was adjusted to pH 6.3. Besides being used for the assay, this ascorbate medium was used as a diluent for the ascorbic acid-containing portion described above. All media, after all additions to tubes, were autoclaved at 112° for 5 min. The inoculum culture consisted of the second of two consecutive serial 24 hr. broth cultures grown in the assay medium supplemented with 1 m μ g. leucovorin/ml.; the culture was centrifuged, resuspended in 5 ml. sterile physiological saline and diluted 1/100 with sterile saline. One drop of this dilution served as inoculum for each assay tube. (Leucovorin was obtained from the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York, U.S.A., in solution in ampoules.) Incubation was for 24 hr. at 35° , and growth was determined turbidimetrically at 600 m μ . For the aerobic *P. cerevisiae* assay a second method, which yielded results almost identical with those given by the first method described, was used; this second assay was performed with the medium and method described in the *Difco Manual* (1953). Assays with *Streptococcus faecalis* were also performed by using the medium and method described in the same publication (*Difco Manual*, 1953). Assays performed with the ascorbic acid-containing portion were treated as described for the *P. cerevisiae* assay.

Assays with *Lactobacillus casei* were made by using the basal medium of Nurmikko (1954); 6.0 ml. media were used per tube. The medium was autoclaved at 112° for 5 min., with the glucose added before autoclaving. The inoculum was prepared like the *Streptococcus faecalis* inoculum. Growth was recorded turbidimetrically after incubation for 60 hr.

In each assay method, standard curves were prepared by using leucovorin, and the results were expressed in terms of leucovorin activity.

Analysis of Streptococcus faecalis culture medium for phenylalanine activity. For production of the phenylalanine-active factor synthesized by *Streptococcus faecalis*, 5.0 ml. of the basal medium, supplemented after autoclaving with 0.05 μ g. PGA/ml., were inoculated from the stock stab culture. After 24 hr. a second transfer was made to the same medium, and after a further 18 hr., one loopful was transferred to 100 ml. of the same medium.

The concentration of the phenylalanine-active factor was accomplished in the following way. After incubation of *Streptococcus faecalis* for 24 hr. in the medium described, the organisms were removed by centrifugation, the supernatant fluid adjusted to pH 6.7 with 2N-NaOH and evaporated to dryness at 60° in a 1 l. beaker. The residue was redissolved in a minimum quantity of water and extracted with

100 ml. 95 % (v/v) ethanol in water, followed by a second extraction with 50 ml. 95 % (v/v) ethanol. The ethanolic extract was evaporated to dryness at 60°, re-dissolved in a minimal amount of water, and preserved at -10° until assayed for activity.

Bioautography of the ethanolic extract for phenylalanine activity was accomplished in the following way, a modification of a method described by Block, Durrum & Zweig (1958). The concentrate was applied in a band to Whatman No. 1 chromatography paper; after drying, it was developed by an ascending technique with the solvent acetone + water + urea (60 + 40 + 0.5; v, v, w). After drying, the paper was again developed with 0.04N-HCl at right angles to the first direction so that the phenylalanine-active factor was concentrated at the top of the paper. The paper was dried and then placed face down on basal medium solidified with 1.5 % (w/v) agar and supplemented with 0.05 µg. PABA/ml., previously seeded with *Lactobacillus plantarum*. The paper strip was removed after 1 hr., and the plates incubated overnight.

Conventional methods of 'spotting' were tried on numerous occasions without success, probably because of the interference with the migration of the test compounds by the high concentration of other solutes in the concentrate. Even with the present method, however, when compounds such as shikimic acid were chromatographed from pure solution, in contrast to being incorporated into the ethanolic extract concentrate, a considerable difference was noticed between the R_f values. When applied from pure solution, the shikimic acid migrated much further than when incorporated in concentrate. Therefore, special care was taken to avoid the comparison of R_f values of pure compounds with the R_f values of the phenylalanine-active factor in the concentrate.

RESULTS

Figure 1 presents the results obtained when various mixtures of phenylalanine, PABA and PGA were supplied for the growth of *Lactobacillus plantarum* and *Streptococcus faecalis*, when grown separately and in mixed culture. It can be seen that syntrophic growth occurred in the medium which lacked phenylalanine and PGA but was supplemented with PABA (designated by code 19 in Fig. 1). In media of the same composition (Fig. 1; codes 5 and 12), neither organism alone grew significantly during the incubation periods recorded. Syntrophic growth was not observed when PABA was not present in the medium.

Growth of *Lactobacillus plantarum* in the absence of phenylalanine eventually occurred upon prolonged incubation. The time at which such growth appeared was quite variable, but generally occurred within 3-5 days. Experiments wherein the size of the inoculum was decimally decreased indicated that after incubation for 120 hr., even those tubes which had been theoretically inoculated with one *L. plantarum* organism eventually exhibited growth in the basal medium supplemented with PABA but lacking phenylalanine. These results indicated that carry-over of phenylalanine in the inoculum or in the organisms of the inoculum was not a factor in this delayed response.

In the present study a direct microscopic counting procedure, as by using the Petroff-Hauser counting chamber, could not be used to follow the growth of each

of the two organisms in syntrophic culture, because of the inability to distinguish microscopically in this technique the organisms of *Lactobacillus plantarum* from those of *Streptococcus faecalis*. On solid media, such as microassay culture agar (BBL), *L. plantarum* was found as long rods; but in the basal medium supplemented with phenylalanine and PABA, the organism occurred as short rods or cocci. In micro-inoculum broth (BBL) stained smears made up to 18 hr. revealed short rods or cocci; after 25 hr. they revealed predominantly long rods.



Fig. 1. The effect of phenylalanine, PABA, and PGA in the nutrition of *L. plantarum* and *S. faecalis* at 35° in a chemically defined medium. The medium to which the compounds were added is the basal medium referred to in the text under Methods. PH = phenylalanine, 200 µg./ml.; PGA = pteroylglutamic acid, 0.01 µg./ml.; PABA = p-aminobenzoic acid, 0.2 µg./ml. Code designations 1 through 21 identify various supplements and inocula.

Trypticase soy agar (BBL) supplemented with 0.25 % (w/v) glucose at pH 9.0 was found to serve as a selective medium for the growth of *Streptococcus faecalis*. The same medium at pH 7.3 supported the growth of both *S. faecalis* and *Lactobacillus plantarum*. The selective medium was used to determine the actual numbers of each organism during a period of syntrophic growth. The results (Table 1) indicate that *S. faecalis* predominated in the culture up to 26 hr., but that after 46 hr. it decreased markedly so that *L. plantarum* eventually predominated. The discrepancies apparent as between the results obtained by the three methods of enumerating the total population can be attributed to: (1) differences in size of organisms at different times; (2) the presence of non-viable, as well as viable, organisms as incubation was prolonged.

Table 1. *Growth, as measured turbidimetrically, microscopically, and by the standard plate count, of Lactobacillus plantarum and Streptococcus faecalis in pure and mixed cultures*

Growth was in basal medium (see Methods) lacking phenylalanine and PGA, and supplemented with 0.002 μ g. PABA/ml. Incubation at 35°.

The standard colony count for *S. faecalis* was determined by plating the syntrophic culture in Trypticase soy agar supplemented with 0.25 % (w/v) glucose and adjusted to pH 9.0.

The total (*S. faecalis* and *L. plantarum*) colony count was determined with the same medium at pH 7.3. The difference between the two counts represented the standard colony count for *L. plantarum*.

Incubation time (hr.)	Pure cultures		Both cultures
	<i>S. faecalis</i>	<i>L. plantarum</i>	
	Optical density (600 m μ)		
22	0.00	0.05	0.37
46	0.00	0.05	1.50
51	0.00	0.06	1.70
74	0.00	1.70	1.70
	Petroff-Hauser total count (cells/ml.)		
22	—	—	1,630,000,000
46	—	—	5,000,000,000
51	—	—	4,500,000,000
74	—	—	9,000,000,000
	Standard plate counts from syntrophic culture		
22	795,000,000	35,000,000	830,000,000
46	30,000,000	2,605,000,000	2,635,000,000
51	< 1,000,000	2,694,000,000	2,694,000,000
74	< 1,000,000	1,830,000,000	1,830,000,000

In preliminary assays for the presence of the phenylalanine-active factor synthesized by *Streptococcus faecalis*, the ethanolic extract of the culture medium from a 24 hr. culture of *S. faecalis*, and the ethanol-insoluble residue remaining after this extraction, were added in different amounts to sterile paper disks; these were placed on the surface of solidified basal medium supplemented with PABA (but lacking phenylalanine), which had been previously seeded with *Lactobacillus plantarum*. The presence of the phenylalanine-active factor was found to reside in the ethanolic extract, as exhibited by the growth response around the corresponding disk. A bioautograph was prepared in the manner previously described, on which the ethanolic extract alone was compared with the extract to which phenylalanine had been added. It was evident that the difference in the positions of phenylalanine and of the phenylalanine factor in the extract ruled out the possibility that the factor was identical with phenylalanine. The factor was a faster moving compound than phenylalanine in the solvent system used.

A second bioautograph was prepared in which pure shikimic acid was compared with extract containing added shikimic acid. The pure shikimic acid was used to demonstrate that it was possible to obtain a 'spot' with the concentration used. However, the appearance of two 'spots' on the system when extract + shikimic acid was tested, indicated that the factor synthesized by *Streptococcus faecalis* was not identical with shikimic acid. The fact that shikimic acid migrated more slowly

when incorporated in the extract, than when applied from pure solution, was also demonstrated. Nevertheless, the appearance of the shikimic acid 'spots', although different in position, clearly identified the known compound. The 'spot' of low density, that of the excreted factor, was similar in position and size to that exhibited in the preceding bioautographic experiment. The difference in positions of the two shikimic acid 'spots' may be attributed to interference with migration of the compound by the high concentration of other solutes in the extract.

The results of microbiological assays of the fractions prepared from the culture medium of *Lactobacillus plantarum* cultures, obtained as previously described, are presented in Table 2. The quantitative aspect of these results has significance with respect to the nature of the folic acid-like compound(s) synthesized and excreted into the medium by *L. plantarum*, in the light of recent developments in the field of folic acid metabolism. This topic is discussed below.

Table 2. *Folic acid activity present in Lactobacillus plantarum culture medium after 24 hr. growth, as assayed by Pediococcus cereviseae, Streptococcus faecalis, and Lactobacillus casei with leucovorin as reference standard*

Organism	Type of assay	Sample no. 1	Sample no. 2
		Equiv. $\mu\text{g.}$ leucovorin/ml. culture fluid	
<i>P. cereviseae</i>	Aerobic	3.7	2.9
	Anaerobic*	14.8	11.6
<i>L. casei</i>	Aerobic	9.9	10.2
<i>S. faecalis</i>	Aerobic	11.2	10.1
	Anaerobic	24.8	22.3

* Anaerobic = addition of 1.33 mg. ascorbic acid/ml. assay medium, with adjustment of medium to pH 6.3.

DISCUSSION

The failure to demonstrate syntrophic growth of *Lactobacillus plantarum* with *Streptococcus faecalis* in the absence of added PABA was in contrast to the results obtained by Nurmikko (1954) who reported syntrophic growth of the two organisms in a medium lacking PABA (designated code 21 in Fig. 1), although the growth was clearly slower than in a medium which lacked phenylalanine and PGA. Only when the purines and uracil were omitted, in addition to PABA, were the two organisms unable to grow together. In the present investigation, the medium contained the purines and uracil; but in the absence of PABA, syntrophic growth did not occur, even on prolonged incubation.

Although growth of *Lactobacillus plantarum* in the presence of phenylalanine, but not of PABA or PGA, did appear after prolonged incubation, within the first 3-5 days independent requirements for phenylalanine and PABA could be demonstrated. This observation is also in contrast to the results of Nurmikko (1954), who reported that the requirement for PABA could be effectively replaced by phenylalanine, that this replacement could be inhibited by sulphonamides, and that increasing concentrations of phenylalanine were effective in overcoming the

inhibiting action of the sulphonamides. When one considers that *L. plantarum* has long been used as an assay organism for PABA in a medium containing Norite-treated acid-hydrolysed casein (Lewis, 1942), it does not appear likely that there could be a replacement of PABA by phenylalanine.

In microbiological assays involving tetrahydroPGA, it has been found that the pH value of the medium is important when the standard curve is based upon response to leucovorin (Donaldson & Keresztesy, 1959). Therefore, at pH 6.3 (the pH value used in the anaerobic assays shown in Table 2), the activity of the oxygen-labile compounds assayed in the *Pediococcus cerevisiae* system, and represented by the difference between the aerobic and anaerobic *P. cerevisiae* assays, may be much higher than is indicated, if tetrahydroPGA be assumed to be the major component of this fraction.

The assumption is made, for purposes of this discussion, that differences in pH value will not affect compounds other than tetrahydroPGA; otherwise, comparison becomes impossible because of the use of different pH values for aerobic and anaerobic assays, or because of the use of different organisms. Therefore, with this assumption, the kinds of folic acid compounds (in a general way only) can be deduced from the results of Table 2. Moreover, the concentrations of each of the different folic acid-like entities synthesized by *Lactobacillus plantarum* can also be determined as summarized in Table 3.

Table 3. *Summary of types of folic acid-like compounds synthesized by Lactobacillus plantarum as calculated from microbiological assay data*

Type of folic acid compounds	Sample no. 1 ($\mu\text{g./ml.}$)	Sample no. 2 ($\mu\text{g./ml.}$)
1. Oxygen-stable, for <i>P. cerevisiae</i> (e.g. leucovorin)	3.7*	2.9*
2. Oxygen-stable and oxygen-labile, for <i>P. cerevisiae</i> (e.g. leucovorin + tetrahydroPGA)	14.8*	11.6*
3. Oxygen-labile, for <i>P. cerevisiae</i> (e.g. tetrahydroPGA)	(14.8 less 3.7) = 11.1†	(11.6 less 2.9) = 8.7†
4. Oxygen-stable, for <i>L. casei</i> (e.g. leucovorin) + PGA	9.9*	10.2*
5. Oxygen-stable, for <i>L. casei</i> but not stimulatory for <i>P. cerevisiae</i> (e.g. PGA)	(9.9 less 3.7) = 6.2†	(10.2 less 2.9) = 7.3†
6. Oxygen-stable, for <i>S. faecalis</i> (e.g. leucovorin) + PGA	11.2*	10.1*
7. Oxygen-stable, for <i>S. faecalis</i> but not stimulatory for <i>P. cerevisiae</i> (e.g. PGA)	(11.2 less 3.7) = 7.5†	(10.2 less 2.9) = 7.2†
8. Oxygen-stable and oxygen-labile, for <i>S. faecalis</i> (e.g. PGA + leucovorin + tetrahydroPGA)	24.8*	22.3*
9. Oxygen-labile, for <i>S. faecalis</i> (e.g. tetrahydroPGA)	(24.8 less 11.2) = 13.6†	(22.3 less 10.1) = 12.2†

* Experimentally determined value (see Table 2). † Calculated from experimental data.

The values presented in Table 3, it may be noted, agree very well with theoretical calculations. For example, the oxygen-stable fraction stimulatory for *Pediococcus cerevisiae* + the oxygen-labile fraction stimulatory for *P. cerevisiae* + the oxygen-stable fraction stimulatory for *Streptococcus faecalis* but not for *P. cerevisiae*, should

be equivalent to the sum total of all the folic acid activity present in the sample. This value was determined experimentally by assay with *S. faecalis*, under anaerobic conditions, to be 24.8 or 22.3 $\mu\text{g./ml.}$ of spent medium (Table 2). This can be determined by the following scheme:

	Sample no. 1 ($\mu\text{g./ml.}$)	Sample no. 2 ($\mu\text{g./ml.}$)
Oxygen-stable fraction	3.7	2.9
Oxygen-labile fraction	11.1	8.7
Oxygen-stable fraction (stimulatory for <i>S. faecalis</i> but not for <i>P. cerevisiae</i>)	7.5	7.2
Total activity of sample on a theoretical basis	22.3	18.8

It may be noted that the values for the oxygen-labile fraction for *P. cerevisiae* and the oxygen-labile fraction for *S. faecalis* (Table 3) are nearly equal, which would indicate that the same chemical entities, e.g. tetrahydroPGA, are being assayed. Since there appears to be little difference between values for the oxygen-stable fraction determined by assay with *Lactobacillus casei* (but having no activity for *P. cerevisiae*) and the oxygen-stable fraction for *S. faecalis* (having no activity for *P. cerevisiae*), it may be concluded that here again the same chemical entities, e.g. PGA, are being assayed.

From these observations, it would appear that three kinds of folic acid compounds are present in 24 hr. spent medium of *Lactobacillus plantarum*: (1) a fraction which is oxygen-stable and supports the growth of *Pediococcus cerevisiae* (such as leucovorin, leucovorin-anhydride, or ^{10}N -formyltetrahydroPGA); (2) a fraction which is oxygen-labile and supports the growth of *P. cerevisiae* (such as tetrahydroPGA or dihydroPGA); (3) a fraction which is oxygen-stable and does not support the growth of *P. cerevisiae* but which is active for *Streptococcus faecalis* and *Lactobacillus casei* (such as PGA, ^{10}N -formylPGA, folylglutamate, thymine, thymidine, ^{10}N -formyldihydroPGA). It would also appear that pteric acid and ^{10}N -formylptericoic acid are not present, since these compounds are active for *S. faecalis* but not for *L. casei*.

The foregoing generalizations cannot be considered as proved by the evidence presented, but merely offer a reasonable explanation based on the recent developments concerning folic acid metabolism. As mentioned, much more information needs to be obtained about the effect of pH value on compounds other than tetrahydroPGA when the response is measured with reference to leucovorin standard curves. In addition, more data are needed about the quantitative response of different assay organisms to these compounds when interpreted in terms of the response of the assay organisms to leucovorin.

The elimination of phenylalanine, as being identical with the factor synthesized by *Streptococcus faecalis*, differs from the observation reported by Judge (1958). However, since Judge used filtrates of cultures which had been concentrated *in vacuo* 5- to 10-fold, and since these materials were applied to chromatograph paper in a single 'spot' in 5-10 $\mu\text{l.}$ quantities, it is quite possible that interference with the migration of amino acids occurred in a manner similar to that found here for shikimic acid, namely, because of a large concentration of other solutes in the concen-

trate. Since ninhydrin was used as the indicator, all α -amino acids should have been detected. The system used in the present investigation used a biological indicator, the growth of *Lactobacillus plantarum*, the other member of the syntrophic pair, which specifically indicated the position of the phenylalanine-active factor on the agar plate.

The demonstration that neither phenylalanine nor shikimic acid was identical with the factor synthesized by *Streptococcus faecalis* and responsible for supporting the growth of *Lactobacillus plantarum*, suggests the possibility that the factor may be one of the compounds which have been suggested or demonstrated as intermediates in the biosynthesis of phenylalanine. Of such compounds (a) tyrosine cannot replace the phenylalanine requirement of *L. plantarum*, and (b) quinic acid and phenyl pyruvic acid cannot replace the phenylalanine requirement of *L. plantarum* (Nurmikko, 1954). Other possibilities about the nature of the substance involved are 5-dehydroshikimic acid, 5-dehydroquinic acid and shikimic acid-5-phosphate; these, however, were not explored.

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The Relationship Between Activity and Cell-wall Permeability in Dried Baker's Yeast

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SUMMARY

A close relationship has been found between the baking activity of dried yeast and the yeast cell volume after reconstitution; other things being equal, the cell volume is in turn dependent upon the permeability of the cell wall. It appears that the total observed loss of activity occasioned by drying yeast may be attributed to increased cell-wall permeability.

INTRODUCTION

In the production of dried baker's yeast a strain of *Saccharomyces cerevisiae* is used and the moisture content is decreased from about 70% to 7-8% by a drying process which may be one of several alternatives. Drum drying, in which the pressed yeast is placed in a revolving drum and dried in a current of hot air, is one method used. The product shows markedly improved storage properties. Whereas pressed yeast loses much of its baking activity after 1 week at room temperature, dried yeast may be stored for several months without appreciable deterioration. When required for use, dried yeast is simply added to the desired quantity of water and a suspension of rehydrated cells is obtained after a few minutes; this process is commonly referred to as reconstitution. Even the most favourable drying conditions, however, cannot produce a dried yeast which retains all the activity which it originally possessed in the pressed state; a greater or lesser proportion of the original activity is lost on drying, the extent of the loss depending on the strain of yeast used, the drying conditions and the method by which the yeast was propagated. As a general rule, the higher the initial activity the greater the loss suffered on drying.

It has been known for some time that dried yeasts become permeable to small molecules and ions, as shown by the work of Meyerhof & Kaplan (1951), Herrera, Peterson, Cooper & Pepler (1956), and Rothstein, Jennings, Dennis & Bruce (1959). While this is suggestive of a connexion between cell permeability and activity, the extent of the relationship has not been investigated. It has indeed been suggested that a decrease in permeability occurs on drying, resulting in a slower release of enzymes to the medium (Proskuryakov & Operysheva, 1956). It is the intention in this paper to show that the relationship between dried-yeast activity and cell-wall permeability is so close as to suggest that the latter is the main determining factor in dried-yeast performance; a rapid method for assessing the over-all permeability of the yeast cell wall is also given.

Throughout the work described here, cell-volume measurements were used as an indication of the over-all permeability of the cell wall. In order to appreciate the validity of this assumption it is necessary to consider the osmotic equilibrium of the

yeast cell. The normal healthy yeast cell consists of a mass of protoplasm enclosed by a structural cell wall together with the plasma membrane. In this respect it resembles most plant cells, although in plants the cell wall is relatively rigid so that when the cell is plasmolysed the membrane detaches itself from the cell wall and shrinks along with the cytoplasm, leaving the cell wall unaltered in size and shape. It has been shown in this laboratory, however, that a linear relationship exists between cell volume and external osmotic pressure up to 10 atmospheres, i.e. there is Hookean behaviour within this range; beyond this point the relationship is non-linear, the cell volume decrease for a given osmotic pressure being less than expected. Nevertheless, the yeast cell volume progressively decreases with increasing osmotic pressure up to at least 25 atmospheres. It is thus assumed that the cell wall is flexible, and that true plasmolysis does not occur, up to an external osmotic pressure of at least 10 atmospheres. Within this range the osmotic pressure inside the cell must always be greater than that outside, equilibrium being established when the external osmotic pressure plus the tension within the cell wall balances the internal osmotic pressure. For any yeast suspended in a given liquid the cell volume will thus be a function of the internal osmotic pressure, this being the integrated effect of all the soluble cell components within the plasma membrane. If the cell now becomes permeable to a certain range of molecular species these will no longer contribute to the internal osmotic pressure and the cell will immediately react and restore equilibrium by assuming a smaller volume with a consequent decrease in cell-wall tension; this change will take place before the molecular species concerned has diffused from the cell. The wider the range of molecular species to which the cytoplasmic membrane becomes permeable, the greater will be the diminution in cell volume. Cell volume may therefore be taken as a measure of the over-all permeability of the membrane.

It was shown by Eirich, Bunzl & Margaretha (1936) that the Einstein equation relating the viscosity of a suspension to the volume fraction of the particles was valid for yeast cells in dilute suspension, i.e. the viscosity is independent of the size distribution. For more concentrated cell suspensions the Einstein equation does not apply, but other workers have produced equations which are claimed to be valid e.g. Mooney (1951), Orr & Blocker (1955), Ting & Luebbbers (1957). These equations all differ to a greater or lesser degree but they all verify the fact that the viscosity is a function of volume fraction only.

It was found in this laboratory that no electroviscous effect was apparent under the conditions used, possibly due to the high cell concentration or to the fact that when commercial baker's yeast is suspended in water a number of ionic species are found to be leached from the cells in small concentrations; the ionic strength is probably sufficient to neutralize the electroviscous effect. A calibration curve relating viscosity and cell volume fraction could then be prepared from which the latter could be directly determined. The average dry matter content (*DM*) of pressed yeast is 27% so this volume was adopted as the standard to which estimations were adjusted, enabling valid comparisons to be made.

The cell volume fraction at 27% *DM* (ϕ_{27}) was obtained from the following relationship

$$\phi_{27} = \frac{V_s \phi_s}{(V_s - E') + W(DM/27 - 1)},$$

where: V_s = vol. of yeast suspension on which measurements were taken; ϕ_s = cell volume fraction of suspension; E' = vol. of water added to prepare suspension; W = wt. of yeast sample used; DM = dry matter content of yeast sample.

Once the values of ϕ_{27} for two different yeast samples, or for the same sample before and after a given treatment, have been determined, the ratio between the values will correspond to the ratio between the mean cell sizes, assuming that the dry matter/cell remains constant.

This method for the determination of cell volumes was evaluated and chosen because of the need for greater precision than may be obtained with the centrifuged cell-volume technique; the accuracy of the viscosity measurement was such that changes in cell volume fraction of $\pm 0.05\%$ could be detected. Moreover, an instantaneous reading is obtained and a continuous record of any progressive change may be made.

METHODS

Measurement of viscosity. Viscosity was measured with a 'Poisemeter' electric viscometer manufactured by Messrs Dobbie McInnes Ltd. (Glasgow). The instrument consists essentially of a constant speed electric motor driving a drag member within the fluid to be measured. Changes in viscous drag cause changes in the current flowing in the motor circuits and these changes are indicated on a scale calibrated in angular degrees. The indication obtained varies approximately as the logarithm of the dynamic viscosity of the fluid being measured. Good reproducibility can be obtained. Errors due to variations in mains voltage or frequency are compensated within the instrument.

Calibration of viscometer. In order to obtain a calibration curve relating scale reading or viscosity to cell-volume fraction it was necessary to determine the external water content of a sample of pressed yeast, since the distribution of water in this material may be such that about 25% of the total is outside the cells (White, 1954). This determination was done by the inulin method (Conway & Downey, 1950*a*). It was known from previous experience that it was desirable to operate within the range of cell volume fractions of 0.50–0.57; above this range the suspension is too viscous to be readily handled, while with more dilute suspensions the sensitivity of viscosity to changes in cell volume diminishes rapidly. Yeast (450 g.) was made into suspension with sufficient water (210 ml.) to give a cell-volume fraction of approximately 0.57. Distilled water was added in 5 ml. amounts and the viscosity read after each addition. All measurements were carried out at 20°. The external water content of the yeast used was found to be 14.3% (v/w), from this and the volume of the suspension the volume fraction could be calculated for each dilution by the relationship $\phi_s = 1 - (E + E')/V_s$, where E = vol. of external water in 450 g. pressed yeast, E' = total vol. of water added at each dilution, and ϕ_s = volume fraction of the suspension.

Preparation of samples. For an accurate viscometer reading about 600 ml. of yeast suspension was required; this was provided by the suspension of 450 g. pressed yeast in 200 ml. water or 200 g. dried yeast in 500 ml. water. These usual quantities were, however, frequently varied for special trials. All samples were brought to 20° before measurement.

Drying technique. The experiments described in this paper were carried out with

a yeast strain of high activity which is not normally used for dried yeast production (standard DCL baker's yeast). This enabled a wide range of activities to be studied since this material was found to give very variable results on drying. Pressed yeast (1000 g.) was forced through a die by means of a hydraulic ram to yield a mass of long, vermicelli-like strands of extruded yeast (1/20 in. diameter), and was then transferred to a drying drum, 16 in. in length and 9 in. diameter, constructed from sheet steel. The drum was rotated at 24 rev./min. and dry air at 50° passed through at a flow rate of 5 foot³/min. The interior of the drum was fitted with three 'flights', each 16 in. × 1½ in., to lift the yeast to near the top of the drum and then allow it to fall through the air stream. During drying the long strands of yeast were rapidly broken down to small pellets. Drying was continued until the moisture content had fallen to 8%, this taking usually about 16 hr.

Examination of the permeability of cells to specific substances. Pressed yeast (450 g.) was suspended in 180 ml. water and adjusted to pH 5.0. The temperature was brought to 20° and the viscosity adjusted to a particular reference point on the viscometer scale by the further addition of water; 600 ml. of this suspension were then measured into a clean dry vessel. The cell-volume fraction of this suspension was then 0.599, i.e. 335 ml. cells and 265 ml. water. Ten ml. of a solution containing such a weight of the substance under examination as to make 275 ml. of 0.25 osmolar solution were pipetted into the suspension and the reading observed after 2 min. and at such intervals thereafter as was considered necessary. The relative cell volume (RCV) i.e. cell volume after addition/cell volume before addition was then obtained from a previously prepared graph. When the substance under examination did not pass through the cell wall it would, of course, exert an osmotic effect and the observed viscosity would be much lower than that calculated for the addition of 10 ml. water alone; moreover, the reading would be constant. For a substance which passed through the cell wall and took a measurable time to do so, the viscosity would show the same initial decrease, but as the substance slowly diffused into the cell the cell volume and viscosity would increase until diffusion equilibrium was attained, when the internal and external concentrations were equal and the RCV had risen to 1.0. Finally, a substance which immediately entered the cell would exert no osmotic action and the relative cell volume would remain at 1.0, the viscosity of the solution decreasing only by the addition of the 10 ml. liquid. A similar procedure was used for dried yeasts.

Determination of yeast activity. For this test the standard fermentometer apparatus as described by Burrows & Harrison (1959) was used. Yeast (1.5 g.) was suspended in about 100 ml. water at 37.5° and allowed to stand for 15 min. at about 18°; 62.5 ml. of 10.8% (w/v) sodium chloride were then added to the suspension and the volume made up to 250 ml. Of this suspension 15 ml. were pipetted into a fermentometer bottle and left for 15–20 min. in the fermentometer bath; 20 g. flour was then added and mixed thoroughly. Thirteen minutes after the addition of flour the system was closed to the atmosphere and the volume of gas evolved after 3 hr. recorded. The observed gas volume was corrected to 20° and 760 mm. Hg. pressure and the yeast activity expressed as ml. gas evolved/g. dry matter.

Determination of yeast dry matter. An accurately weighed sample of about 1 g. was dried at 105° for 6 hr. in a laboratory oven and re-weighed after cooling in a desiccator. The dry matter was calculated from the loss in weight.

RESULTS

Comparison between pressed and dried yeasts

Cell volume. A microscopic examination of the cells of pressed yeast before drying and of the cells as reconstituted after drying revealed no morphological difference other than the fact that the cells after drying appeared smaller. This observation was confirmed by measurement of the cell-volume fraction. This was found to be 0.811 for a sample of pressed yeast; after drying to 8% moisture and reconstitution, a ϕ_{27} value of 0.526 was obtained. Hence the mean cell volume after drying was only 65% of that before drying.

Effect of sodium chloride. Two reasons for the decrease in cell volume on drying seemed possible: either an increase in the permeability of the cell wall or an alteration of the mechanical properties of the wall may have occurred. To distinguish between these possibilities it was necessary to examine directly the permeability of dried yeast. It is well known that the inner region of the yeast cell wall is impermeable to sodium chloride under normal circumstances although the compound will permeate throughout the outer region (cf. Conway & Downey, 1950*b*; Malm, 1947; Kotyk & Kleinzeller, 1958). This was verified by the addition of sodium chloride to a sample of standard yeast cream to make a 0.125M-solution in the external water. The relative cell volumes obtained were 0.9543, 0.9540 and 0.9530 for three different yeast samples, the viscosity remaining constant for periods up to at least 24 hr. This experiment was repeated on two samples of dried yeast, reconstituted at 37.5°, from different laboratory dryings. With both samples it was found that the RCV remained at 1.0, apparently indicating that the sodium chloride passed immediately through the cell wall. It could still be argued, however, that the mechanical properties of the cell wall had been altered by drying to such an extent that it was capable of withstanding the osmotic forces. To test this possibility, the extent to which the sodium chloride had penetrated the cell was estimated directly by titration of the filtrate from a cell suspension containing a known weight of sodium chloride. From a knowledge of the cell-volume fraction it was readily calculated that the salt had penetrated through 60% of the cell volume, assuming that only simple diffusion was involved.

Permeation by other substances. The effect of a number of other substances on reconstituted dried yeast was examined. These included: urea, thiourea, malonamide, acetamide, glucose, sucrose, hexamethylenetetramine, peptone. Each of these substances was found to pass directly through the cell wall, whereas in the case of pressed yeast they either did not penetrate the wall or passed through at a relatively slow and measurable rate. It was of interest to note that the cell wall of the strain of pressed yeast used was completely impermeable to hexamethylenetetramine, even after prolonged exposure; Ørskov (1945) found that this substance diffused slowly through the cell wall of a different strain. This indicates that different strains of yeast can show different permeability relationships.

Reconstitution of dried yeast at different temperatures

The activity of dried yeast is greatly influenced by the temperature of reconstitution (Peppler & Rudert, 1953), 37.5° being considered the optimum by many authorities, although this value is not critical within a few degrees. Two identical

samples of dried yeast were reconstituted, one at 37.5° and the other at 8°. The values of ϕ_{27} were found to be 0.582 and 0.513, respectively, as compared with 0.801 for the same yeast before drying. The permeability to urea of the original yeast and of the two reconstituted samples was also examined. It was found that diffusion equilibrium was reached in 9 hr. with the pressed yeast, 1.5 hr. with the sample reconstituted at 37.5°, while diffusion equilibrium was instantaneous with the final sample reconstituted at 8°.

Autofermentation

It was found that the reconstitution of dried yeast in the quantity and concentration required for viscosity determination gave rise to vigorous autofermentation which persisted for 1 to 2 hr. Moreover, although no measurements of gas evolution were taken, it was obvious that the more active the yeast, the more vigorous the fermentation. Viscometer measurements were normally postponed until gassing had ceased. The substrate for this autofermentation is not known, but no fermentable carbohydrate was detected in the filtrate from a freshly reconstituted dried yeast. It appears that the increased permeability of the cell wall creates a certain amount of disorder within the cell and in the cell-wall region, whereby enzymes are allowed to come into contact with substrates which would not normally be available in the intact healthy cell. The view held is that a 'perfect' dried yeast would show no autofermentation; slightly damaged yeasts, however, exhibit vigorous autofermentation, indicating that there is still a good capacity for fermentation although the disarrangement of the cell is sufficient to bring normally separated enzymes and substrates into close proximity. As the amount of damage becomes more extensive, the over-all fermentative capability decreases through the loss and dilution of essential co-enzymes etc., and this effect more than counterbalances the increased substrate availability. By the use of iodoacetic acid as an inhibitor of the autofermentation it was shown that a decrease in the permeability of the cell wall took place during this period. Dried yeast was reconstituted at 37.5° in water containing 0.2% (w/v) iodoacetic acid; the ϕ_{27} value was 0.571. A further sample of the same yeast was reconstituted in the normal manner and 0.2% (w/v) iodoacetic acid added to this suspension after autofermentation had ceased; the ϕ_{27} value was then 0.637. The two samples were examined for permeability to urea in the usual way. It was found that the cell permeability corresponded to the difference in cell volumes, diffusion equilibrium taking about 1 hr. for the sample to which iodoacetic acid was added immediately, and 4 to 5 hr. for the other.

Relationship between cell volume and activity

The determination of the cell volume and permeability of a number of dried yeast samples showed that they did not all behave in the manner previously described, i.e. were fully permeable to all substances for which measurement was possible. This was found to apply only to yeasts of very poor quality. Yeast samples of higher activity were examined as they became available and it was found that they exhibited a variety of permeability effects according to their activity. Very active samples were completely impermeable to sodium chloride but permitted the passage of urea fairly rapidly; other samples of lesser activity were almost instantaneously permeable to urea and allowed sodium chloride to diffuse at a measurable

rate. It had been hoped to find some substance which could be used in a standard permeability test and which would show a measurable but variable permeation rate with all yeast samples, i.e. never becoming zero or infinite with any sample. It became apparent, however, that such an ideal could not be attained and it was at this stage that the cell volume was adopted as a measure of the over-all permeability of the cell.

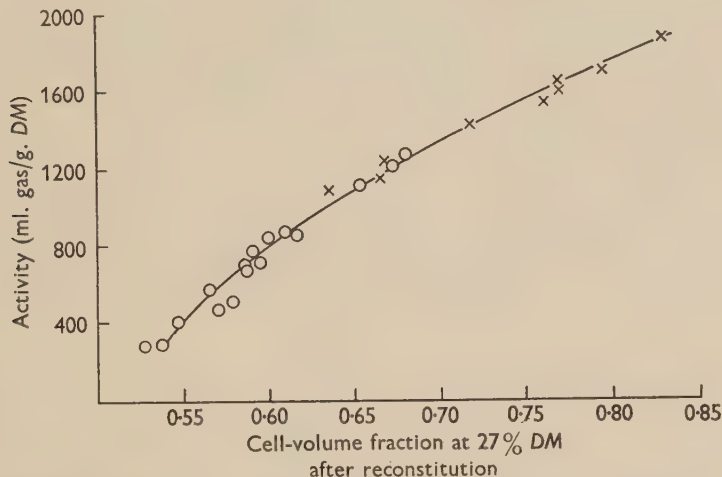


Fig. 1. Relationship between activity of pressed yeast and cell-volume fraction at 27 % dry matter (DM) after reconstitution. O, Fully dried samples; X, samples removed at intervals during drying.

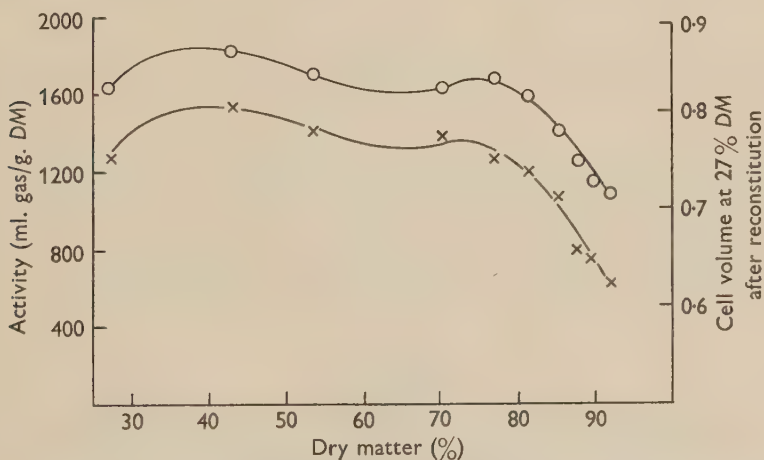


Fig. 2. Relationship between activity, cell-volume fraction and dry matter during the course of drying. O, yeast activity; X, cell-volume fraction at 27 % dry matter (DM) after reconstitution.

A number of laboratory dryings of different batches of yeast were carried out and the dried products examined for cell volume and activity. The results of this series are given in Fig. 1 (circled points) where it will be seen that there is a very close relationship between these variables. As an extension of this, the cell volume and activity were measured at intervals during the drying process in one case (Fig. 2).

The cell volume curve was found to be an exact duplicate of the activity curve. When these results were transferred to Fig. 1 (crosses) they formed a smooth extension to the curve already obtained.

To prove that the diminution in cell volume and the increased permeability of the cell were not merely incidental consequences of the loss in activity, a number of identical samples of cream prepared from pressed yeast were incubated for various times at 45° and the cell-volume fraction then measured after cooling to 20°. A portion of each suspension was filtered after incubation and the activity and dry matter of the pressed samples determined. It was shown thereby that the activity of yeast could be almost completely destroyed without the cell volume being significantly affected (Fig. 3).

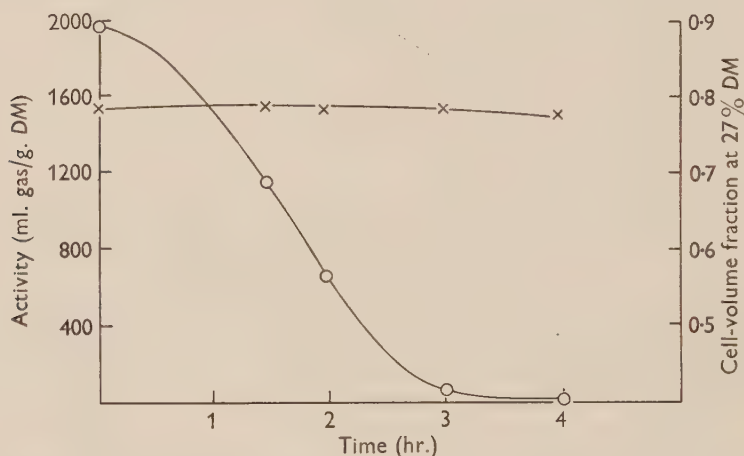


Fig. 3. Effect of incubation of yeast cream at 45°. O, yeast activity; ×, cell volume fraction at 27% dry matter (DM).

Throughout the investigations mentioned in this paper, the pH value of reconstituted dried yeast suspensions was routinely measured. There was a fairly good correlation between pH value and activity; suspensions of poor dried yeasts had a pH value of 5.9–6.2 while the better yeasts gave suspensions of lower pH value. This observation is a further reflection of the increased permeability of poor dried yeasts, since the internal pH value of the strain used is about 6.0–6.2.

DISCUSSION

The results presented here serve to illustrate the fact that the permeability of the cell wall is of primary importance in dried yeast research. From the evidence collected it does not appear that any other hypothesis need be invoked to account for the disparity between the activity before and after drying. The correlation between activity and cell volume is sufficiently good to indicate that the former is a function principally of the latter. It was originally thought that heat inactivation of enzymes might play a part, but it seems probable that such an effect is insignificant as compared with the activity loss brought about by increased permeability of cell wall. The view now held is that during drying, no matter how low the moisture content may be diminished, there is no damage to the fermentation system of the

yeast provided that the drying temperature is not excessively high. From approximately 80 % dry matter onwards, however, there is a progressive disarrangement of the cytoplasmic membrane. The damage only becomes apparent when the yeast is resuspended in water and loss of essential components occurs.

The work of Herrera *et al.* (1956) is of particular interest in relation to the results presented here. In studying the differences in activity caused by reconstituting dried yeast at various temperatures it was found that 75 % of the cell diphosphopyridine nucleotide (DPN) was extracted at 4–5°, as compared with 15 % at 43°. These authors suggested that the difference might be accounted for in terms of the rate of rehydration of the cytoplasmic membrane, this process being slow at a low temperature and comparatively fast at the higher temperature of 43°. It is possible that the increase in cell volume reported here as occurring during the autofermentation period was the final stage of such a process. It is to be hoped that future work may yield more definite information about the composition and physical state of the cytoplasmic membrane; this knowledge would be of great value in solving problems associated with drying yeast and other micro-organisms.

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The Sodium and Potassium Content of Non-Halophilic Bacteria in Relation to Salt Tolerance

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SUMMARY

The sodium and potassium contents of cells of 32 strains of non-halophilic bacteria were determined after growth in a standard medium of low salt content. The salt tolerance of each strain was assessed by determining the lowest water activity at which growth occurred in sodium chloride medium. Among the strains examined the potassium contents varied about fivefold and were positively correlated with salt tolerance.

INTRODUCTION

Bacteria differ greatly in ability to grow in high concentrations of sodium chloride. The growth of some strains is prevented by 2-3% salt, but some halophilic bacteria proliferate in media saturated with sodium chloride. These halophiles were shown by Baxter & Gibbons (1956) to contain enzymes which were active in solutions of very high ionic strength. No explanation has been advanced for the large differences in salt tolerance found among non-halophilic bacteria.

Cells of *Salmonella oranienburg* respiring in concentrated solutions accumulated much more potassium than in dilute media (Christian, 1955), suggesting that potassium largely controlled the water activity (a_w) within the cell. Further, the salmonellas are relatively intolerant of high salt concentrations or low a_w (Christian & Scott, 1953) and contain little potassium (Barber, 1931; Christian, 1958), while the staphylococci are very salt tolerant (Maitland & Martyn, 1948; Scott, 1953) and are very rich in potassium (Barber, 1931). The present paper presents results which support the hypothesis that salt tolerance in non-halophiles is related to ability to accumulate potassium within the cells.

METHODS

Organisms. Thirty-two strains of bacteria were studied, comprising: twelve Gram-positive cocci (*Leuconostoc citrovorum*; *Micrococcus lysodeikticus*, *M. roseus*; two *Sarcina* spp.; *Staphylococcus aureus*, *Staph. albus*, *Staph. citreus*; *Streptococcus cremoris*, *Strep. faecalis*, *Strep. lactis* and an unidentified coccus); fifteen Gram-positive spore-forming rods (two *Bacillus cereus*, *B. megaterium*, *B. mycoides*, *B. subtilis* var. *niger*, nine unidentified strains); five Gram-negative strains (*Escherichia coli*, *Flavobacterium* sp., *Pseudomonas fluorescens*, *Salmonella oranienburg*, *Vibrio metchnikovi*).

Salt tolerance. Salt tolerance was determined at pH 7.0 in brain + heart infusion

broth, sodium chloride being added to provide the desired values of a_w in steps of 0.01. Ten ml. volumes of medium were dispensed in 100 ml. screw-cap bottles which, after inoculation with 0.02 ml. of a 16 hr. broth culture, were incubated at 30° for 28 days. The lowest a_w value which supported growth was determined by observation of turbidity.

Ion content. For analysis, organisms were grown in 100 ml. brain + heart broth (0.993 a_w) in 250 ml. Erlenmeyer flasks shaken at 30°. On reaching the stationary phase of growth, each culture was centrifuged to give two equal cell pellets. One pellet was weighed before and after drying at 105° (giving dry weight and water content) and the other pellet was extracted with cold trichloroacetic acid. Extracts were analysed for sodium and potassium by flame photometry.

Interstitial space was determined by phosphate dilution (Mitchell & Moyle, 1956) in centrifugates of four Gram-positive cocci, one Gram-positive rod and one Gram-negative rod. Large differences were found, probably because of differences in packing fractions as between strains. However, when internal water was calculated from these values, the mean and standard error for the six strains was 1.50 ± 0.03 ml./g. dry weight. This figure was applied to all strains, and, the dry weight and total water content of the centrifugate and the water content of the medium for each preparation being known, the interstitial volume could be calculated. The supernatant fluid, and hence the interstitial medium, contained 163 m-equiv. sodium and 25 m-equiv. potassium/l. The amounts of these ions in the interstitial volume were calculated and subtracted from the values for whole pellets. These net values for sodium and potassium contents were expressed as μ -equiv./100 mg. dry wt. cells. All values presented are means from at least two experiments.

RESULTS

The relationship between the potassium content of cells grown in standard medium and the lowest a_w value which supported growth of the same strains in media adjusted with sodium chloride is shown in Fig. 1. For the thirty-two strains the correlation coefficient was -0.89 ($P < 0.001$). An unidentified coccus, growing to $0.84a_w$, was much more salt-tolerant than the staphylococci (lower limits of 0.89 to $0.88a_w$) yet contained no more potassium. If this organism was excluded the correlation coefficient was -0.91 ($P < 0.001$). Potassium content was directly related to salt tolerance, increasing by about fivefold as the minimum a_w supporting growth decreased from 0.975 to 0.84.

An inverse relationship often exists between cellular contents of sodium and potassium. The increase in salt tolerance which accompanied enhanced potassium content might thus be associated with a decrease in cell sodium. This was generally the case for Gram-positive cocci and Gram-negative rods (Fig. 2*a*). However, at a given sodium content, the rods were less salt tolerant than the cocci. The most salt-tolerant coccus again behaved exceptionally. On the other hand, in Gram-positive sporing rods, sodium content increased appreciably with decrease in limiting a_w value (Fig. 2*b*). The pronounced scatter of values in Fig. 2*b* may result from uptake of sodium during harvesting and preparation for analysis. When only the lowest sodium contents recorded for each strain are plotted, the increase in sodium with decreasing a_w value is more marked. Figure 2 shows also that some strains had internal sodium concentrations lower than in the growth medium. These strains

were amongst the most salt-tolerant cocci and Gram-negative rods, and among the least tolerant of the *Bacillus* spp.

The differences between the two groups of bacteria are further demonstrated in Fig. 3, where potassium to sodium ratios are plotted as a function of the minimum a_w value supporting growth. The steep rise in this ratio as salt tolerance increased in cocci and Gram-negative rods contrasts with its virtual constancy for *Bacillus* spp.

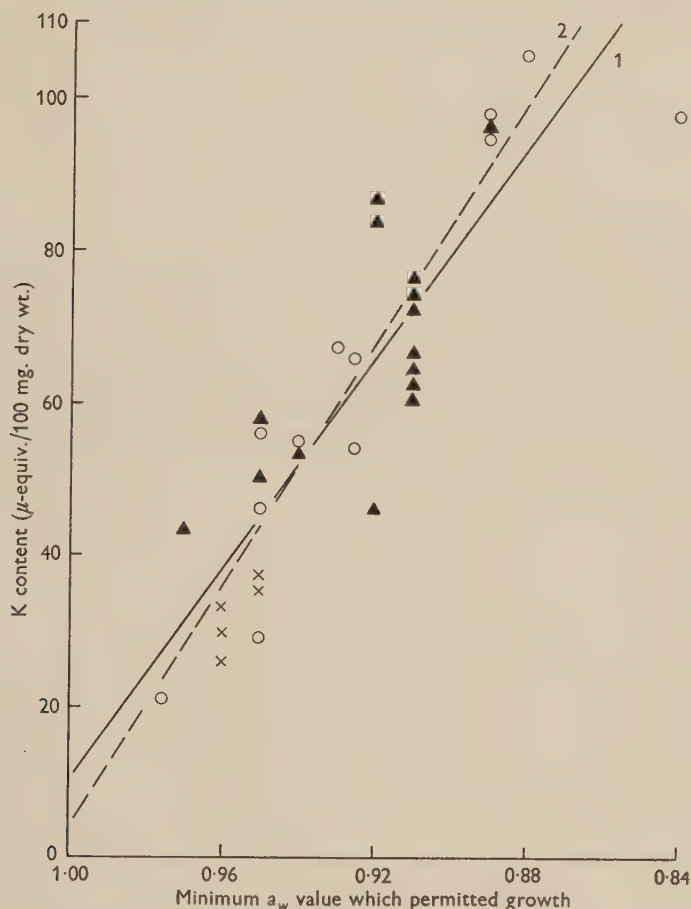


Fig. 1. Potassium content of bacteria in relation to the minimum a_w value which permitted growth. Analyses were performed on organisms grown in basal medium ($0.998a_w$) under standard conditions, and the minimum a_w values for growth were determined in basal medium adjusted to the desired a_w value by addition of sodium chloride. O, Gram-positive cocci; ▲, *Bacillus* spp.; ×, Gram-negative rods. (1) Regression line for all organisms; (2) regression line excluding data for the most salt-tolerant coccus.

Table 1 shows the concentration gradients of sodium and potassium across the cell boundaries of two cocci which differed greatly in salt tolerance. Internal concentrations were calculated on the basis of 1.50 ml. cell water/g. dry wt. It is clear that between strains the internal concentrations of each ion may vary greatly with respect to the external concentration of the same ion and with respect to the internal concentration of the other.

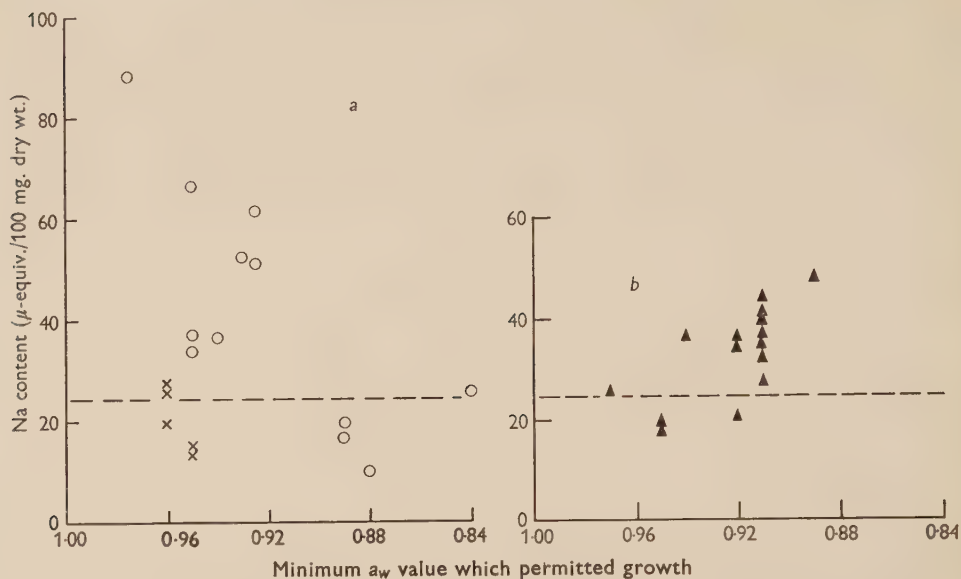


Fig. 2. Sodium content of bacteria in relation to the minimum a_w value which permitted growth. Conditions as for Fig. 1. The broken line indicates the value at which concentrations of sodium in cells and basal medium were equal. (a) O, Gram-positive cocci; x, Gram-negative rods. (b) ▲, *Bacillus* spp.

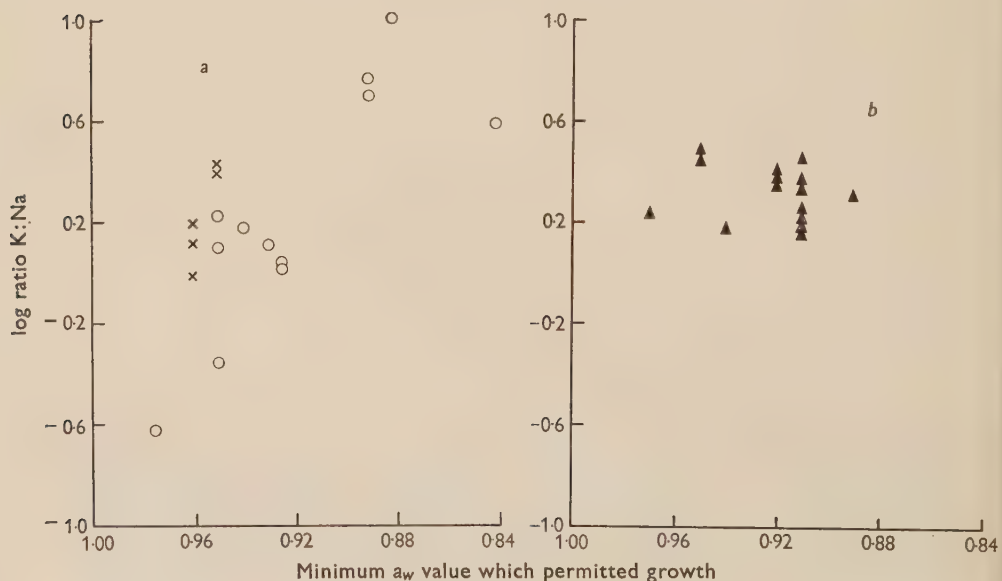


Fig. 3. Logarithms of potassium:sodium ratios in bacteria in relation to the minimum a_w values which permitted growth. Conditions as for Fig. 1. (a) O, Gram-positive cocci; x, Gram-negative rods. (b) ▲, *Bacillus* spp.

Table 1. *Apparent concentrations of sodium and potassium in cocci which differed greatly in salt tolerance*

Organism	Minimum a_w for growth	Sodium concentration (mM)		Ratio cell Na medium Na	Potassium concentration (mM)		Ratio cell K medium K	Ratio cell K cell Na
		cells*	medium		cells*	medium		
<i>Leuconostoc citrovorum</i>	0.975	586	163	3.6	140	25	5.6	0.34
<i>Staphylococcus aureus</i>	0.88	64	163	0.39	708	25	28.3	11.1

* Concentrations calculated on the basis of 1.5 ml. internal water/g. dry wt. cells.

DISCUSSION

The correlation observed between potassium content and salt tolerance holds for such a wide range of bacterial strains that it seems likely to be general among non-halophilic species. However, it must be stressed that this study has been confined to strains growing under one set of environmental conditions. The relationship may not hold for yeasts, since Takada (1956) found that after growth in low-salt medium a yeast adapted to tolerate high salt concentrations contained no more potassium (or sodium) than the parent strain.

The unidentified coccus grew at a sodium chloride concentration above 4M. Such salt tolerance is unusual in a non-halophilic bacterium, and exceeds that of the halophilic *Vibrio costicolus* (Smith, 1937). The water relations of this coccus deserve further study.

Two assumptions were made in calculating net sodium and potassium contents. The barriers to the movement of sodium, potassium and phosphate were assumed to be identical, and a value of 1.5 ml. internal water/g. dry wt. was applied in determining interstitial space for all preparations. Insufficient amounts of potassium were present in the interstitial space for the results to be affected appreciably by errors in these assumptions, but estimates of net sodium content might be subject to considerable variation. However, this would not alter the distribution of strains into those containing higher or lower concentrations of sodium than existed in the growth medium, nor would it affect the conclusion that the lowest sodium contents were found in the most salt-tolerant cocci and the least salt-tolerant *Bacillus* spp. The different relationships between sodium content and salt tolerance in cocci and Gram-positive rods suggest that sodium content is not important for salt tolerance in non-halophilic bacteria. This may be related to the finding that in *Salmonella oranienburg* maintenance of the internal value for sodium was not dependent on the a_w value of the environment, while retention of potassium and of substances which absorbed in the ultraviolet region demanded a relatively low a_w value (Christian, 1958), as did retention of amino acids by *Escherichia coli* (Britten, 1956). The basis of the relationship between potassium content and salt tolerance is obscure. If a high potassium content implies a high concentration of internal solutes, salt-tolerant bacteria will possess low internal a_w . This property would impart resistance to dehydration and plasmolysis, thus overcoming one of the deleterious effects of a concentrated salt environment.

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***In vivo* Studies of Methanogenesis in the Bovine Rumen: Dissimilation of Acetate**

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SUMMARY

The introduction of sodium acetate-2-¹⁴C into a bovine rumen resulted in the *in vivo* labelling of the rumen gases and volatile fatty acids. The relative isotope concentration in substrate and products indicated that a maximum of 5.6% of the methane and 11% of the carbon dioxide in rumen gas might have been derived from the methyl carbon of acetate when the substrate was added to the rumen 18 hr. after the animal had been fed a normal ration. A maximum of 3.2% of the methane and 4.2% of the carbon dioxide might have been derived from the methyl carbon of sodium acetate-2-¹⁴C when this substrate was introduced into the rumen immediately after the animal had been fed. The addition of sodium acetate-1-¹⁴C to the rumen 18 hr. after feeding indicated that 2% of the methane and 10% of the carbon dioxide was derived from the carboxyl carbon of the substrate. Most of the derived radioactivity of the volatile acids of the rumen was found in the butyric acid fraction, although smaller amounts appeared in propionic acid and the volatile fatty acids with a chain length of greater than 4 carbons.

INTRODUCTION

The biochemical reactions mediated by the known species of methane bacteria (Barker, 1956) and the abundance of various methanogenic substrates in rumen fluid suggest that several pathways may be involved in rumen methane formation, as illustrated in Fig. 1. Beijer (1952) detected a *Methanosarcina* sp. in the goat rumen, and Oppermann, Nelson & Brown (1957) demonstrated *Methanobacterium formicum* and a methanogenic acetate-utilizer biochemically resembling *Methanobacterium söhngenii* in enrichment cultures from cattle rumen contents. Nelson, Oppermann & Brown (1958) reported that rumen enrichment cultures stabilized to valeric or butyric acid contained methane bacteria which biochemically resembled *Methanobacterium suboxydans*. Smith & Hungate (1958) isolated and characterized a new species *Methanobacterium ruminantium* which is present in the bovine rumen in great abundance. Thus, with the exception of the oxidation of propionic acid, there is evidence that the rumen contains bacteria capable of generating methane by the pathways shown in Fig. 1. The numerous unsuccessful attempts to obtain the utilization of propionic acid by rumen enrichment cultures (Oppermann *et al.* 1957; Nelson *et al.* 1958) are consistent with the observations of McNeill & Brown (1954), and strongly suggest that propionate is not a substrate for rumen methanogenesis.

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The abundance of evidence relative to the *in vitro* reduction of carbon dioxide to methane by rumen organisms (Carroll & Hungate, 1955; Smith & Hungate, 1958) and the efficiency of the *in vivo* reduction of carbon dioxide to methane by the rumen microflora (Kleiber, 1953) support the hypothesis that hydrogen and carbon dioxide are the chief substrates which give rise to methane in the rumen. However, the existence of acetate-utilizing methane bacteria in the rumen (Beijer, 1952; Oppermann *et al.* 1957; Nelson *et al.* 1958; Yahiro, 1959) suggests that some methane may arise from this source as it does in the anaerobic fermentation of certain industrial wastes (Buswell, 1936). The present investigation was conducted to measure the extent of the *in vivo* conversion of acetate to methane in the bovine rumen.

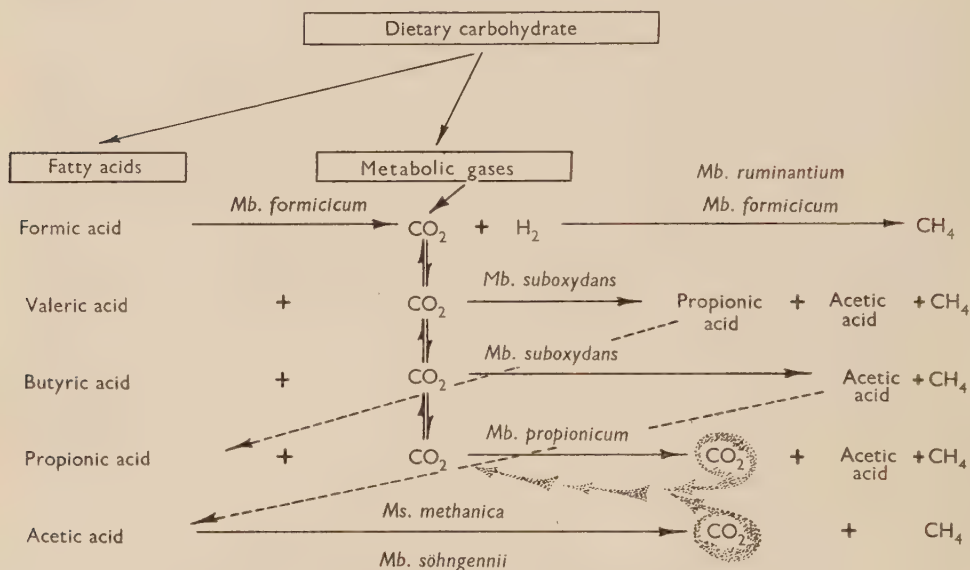


Fig. 1. Theoretical pathways of rumen methanogenesis.

METHODS

In vivo methods. The dissimilation of acetic acid with concomitant methanogenesis was studied in a rumen-fistulated dairy cow maintained on a daily intake of 24 lb. of alfalfa hay and 5 lb. of a 14% protein concentrate mix. The apparatus used for the collection of rumen fluid and gas samples is schematically represented in Fig. 2. Samples of the rumen gas were forced into the Douglas bag by normal rumen contractions. Liquid samples were obtained by use of a sidearm test tube and an aspirator bulb.

The radioactive substrate was added to the rumen 18 hr. after the animal had been fed the standard roughage + concentrate ration, except in one trial as will be noted. This period in the rumen digestion cycle was postulated to be the most favourable for acetate utilization because: (a) the approximate 1:1 ratio of carbon dioxide to methane found at this period (Nelson, Brown & Kingwill, 1960) approaches the actual (Oppermann *et al.* 1957) and theoretical (Buswell, 1936) composition of gas obtained from the methanogenic dissimilation of acetate; (b) the

acetate concentration in rumen fluid is relatively constant at this period (Brown, 1954); (c) formate and carbon dioxide, which may be preferentially utilized and/or be inhibitory to acetate utilization (Oppermann *et al.* 1957), would be at negligible (formate) and minimum (carbon dioxide) concentrations.

Sodium acetate- ^{14}C , labelled in either the 1 or 2 position, was dissolved in 500 ml. distilled water and added to the rumen through the fluid sampling line. The line then was flushed with from 1 to 1.5 l. distilled water. An evacuated Douglas bag was connected to the gas-sampling line and the gas collected. At the end of each time period, the bag containing the sample was removed and an evacuated bag attached.

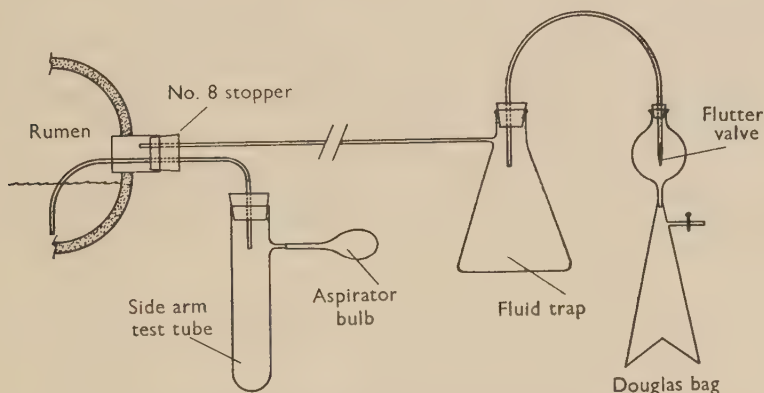


Fig. 2. Schematic representation of apparatus for collection of samples of rumen gas and fluid.

The sodium acetate-2- ^{14}C was purchased from commercial sources and the sodium acetate-1- ^{14}C was synthesized via carbonation of methyl magnesium iodide in the Radiocarbon Laboratory of the University of Illinois.

Chemical methods. A 50 ml. sample of the rumen gas was analysed by absorption-combustion procedures in a Burrell (Model 35-802) apparatus before fractionation of the remainder of the sample in a gas absorption chain. Carbon dioxide was trapped in a 0.25N solution of Primene 81-R (a mixture of tertiary alkyl primary long chain C_{12} - C_{14} amines; Rohm & Hass Co., Philadelphia, Pa.) in methanol (Oppermann, Nystrom, Nelson & Brown, 1959) contained in a large test tube fitted with a sintered glass gas-diffusion tube. Oxygen was absorbed in the second vessel which contained alkaline pyrogallol prepared by dissolving 15 g. pyrogallol acid and 113 g. potassium hydroxide in 100 ml. water. The effluent gas from the absorption train, containing primarily methane and some nitrogen, was subjected to catalytic combustion in the Burrell apparatus.

The carbon dioxide derived from catalytic combustion of methane was absorbed in 0.25M-Primene contained in a special vessel attached to the Burrell apparatus. The gas was passed through the vessel repeatedly until complete absorption had taken place. The volume of carbon dioxide absorbed was recorded and the % methane present in the original gas sample calculated.

Volatile fatty acids (VFA) present in the rumen were determined by a modification of the chromatographic method of Neish (1949) as described in a previous

report by Oppermann *et al.* (1957). The rumen fluid samples were preserved for analysis by acidification with 10N-sulphuric acid and were immediately frozen for storage until analysis could be made.

Carbon-¹⁴C determinations. When rumen fluid samples were chromatographed, three 1 ml. samples were taken, before titration, from three successive 5 ml. samples collected from the top of each volatile fatty acid peak and placed in 13 ml. of scintillation fluid. The remainder of the eluate was titrated and the amount of fatty acid present calculated on the basis of the original volume. The scintillation fluid was prepared by dissolving 3 g. 2:5-diphenyloxazole (PPO) in a litre of high purity toluene. The fatty acid solutions were counted at 810 V. (tap 3) using discriminator settings of 10–50 V. and 50–100 V. on a Tri-Carb liquid scintillation spectrometer (Model 314X, Packard Instrument Co., LaGrange, Ill.).

The absorbent for all carbon dioxide-¹⁴C samples was either 0.25M or M-Primene 81-R. Usually 1 ml. of absorbent solution containing the trapped carbon dioxide-¹⁴C was added to 13 ml. of scintillation fluid and counted at 890 V. (tap 4) using discriminator settings for 10–50 V. and 50–100 V. in the scintillation counter. Carbon dioxide-¹⁴C was determined at a higher tap number than the fatty acids because of the quenching caused by methanol in the Primene solutions (Oppermann *et al.* 1959).

Table 1. *Distribution of ¹⁴C from sodium acetate-2-¹⁴C added* to the rumen 18 hr. after feeding. Trial 1*

Time after addition of ¹⁴ C (hr.)	Volatile fatty acids						Gases			
	Acetate		Propionate		Butyrate		CO ₂		CH ₄	
	(m-mole/ 100 ml.)		(m-mole/ 100 ml.)		(m-mole/ 100 ml.)		CO ₂		CH ₄	
	(SA)†		(SA)		(SA)		(SA)	(%)‡	(SA)	(%)
$\frac{1}{2}$	629§	3.65	0	0.71	56	0.81	—	—	—	—
1	479	3.70	0	0.68	72	0.77	8.7	1.82	1.1	0.24
2	370	3.49	31	0.60	115	0.74	8.9	2.28	3.5	0.91
3	398	3.09	37	0.49	163	0.72	19.5	4.91	5.5	1.38
4	448	3.49	55	0.60	150	0.74	19.2	4.31	7.5	1.68
5	332	3.05	56	0.58	146	0.76	21.6	6.50	12.6	3.80
6	296	2.49	57	0.49	125	0.48	8.8	2.98	4.1	1.39

* 1 mc. of sodium acetate-2-¹⁴C (SA 0.74 mc./m-mole).

† Specific activity = $\frac{\text{Dis./min.}}{\text{m-mole}} \div 1000$.

‡ Percentage derived from substrate = $\frac{\text{SA CO}_2 \text{ or CH}_4}{\text{SA acetate}} \times 100$.

§ Each value mean of three or more observations.

RESULTS

In the first trial 1 millicurie (mc.) of sodium acetate-2-¹⁴C having a specific activity (SA) of 0.74 mc./mmole was introduced into the rumen 18 hr. after feeding, and samples of rumen fluid and gas were collected hourly over a 6 hr. period. As shown in Table 1, the methane and carbon dioxide, as well as propionic and butyric acid became labelled soon after the addition of the acetate-2-¹⁴C. The specific activity and the total concentration of the acetate decreased at a moderate rate throughout

the trial. The amount of ^{14}C in propionic acid increased slightly while the total quantity of this acid decreased slowly. Carbon- ^{14}C concentration in butyric acid increased during the first 3 hr. and decreased gradually thereafter. The total amount of butyric acid in the rumen fluid remained relatively constant during the first 5 hr. before dropping sharply during the sixth hour. The specific activity of the carbon dioxide and methane in the rumen gas increased during most of the experimental period. As the specific activity of each gas gradually increased the % of the gas derived from acetate also increased.

Although the values of radioactivity encountered in the volatile fatty acids, carbon dioxide, and methane were somewhat lower in the second trial, the data followed the same general pattern. However, as shown in Table 2, radioactivity was not detected in the propionic acid fraction and ^{14}C was detected in butyric acid only in the early median portion of the trial.

Although these experiments show that some of the label from acetate was recovered *in vivo* in rumen methane, the simultaneous labelling of propionic and butyric acid and carbon dioxide complicated the elucidation of the role of acetate in methane production because the methane- ^{14}C could have been derived by reduction of $^{14}\text{CO}_2$, or by direct cleavage of the acetate-2- ^{14}C , or both.

The calculation of the relative isotope concentration (Block, Clark & Harary, 1954) or % conversion, i.e.:

$$\frac{\text{specific activity methane-}^{14}\text{C at } T \text{ time}}{\text{specific activity acetate-2-}^{14}\text{C at } T \text{ time}} \times 100 = \% \text{ conversion,}$$

shows that maximum values of only 3.8 and 5.6 % of the methane in trials 1 and 2, respectively, might have been derived from the methyl group of acetate even if it be assumed that labelled butyric acid and/or carbon dioxide- ^{14}C did not participate in the methanogenesis, and that all of the methane- ^{14}C recovered arose from the

Table 2. *Distribution of ^{14}C from sodium acetate-2- ^{14}C added* to the rumen 18 hr. after feeding. Trial 2*

Time after addition of ^{14}C (hr.)	Volatile fatty acids						Gases			
	Acetate		Propionate		Butyrate		CO ₂		CH ₄	
	(m-mole/ 100 ml.)		(m-mole/ 100 ml.)		(m-mole/ 100 ml.)		(SA) (%)‡		(SA) (%)	
	(SA)†		(SA)		(SA)		(SA)		(SA)	
½	439§	3.43	0	0.84	0	0.50	1.6	0.36	1.1	0.25
1	439	3.41	0	0.91	99	0.37	2.2	0.52	0.3	0.06
2	403	2.53	0	1.07	132	0.41	4.2	1.04	1.5	0.37
3	330	3.28	0	0.85	179	0.34	6.8	2.06	3.9	1.19
4	245	3.19	0	0.85	139	0.40	3.8	1.54	9.0	3.66
5	186	2.86	0	0.69	0	0.44	12.2	6.55	1.1	0.60
6	176	2.09	0	0.84	0	0.27	10.5	5.94	1.5	0.88
7	152	3.11	0	0.78	0	0.38	16.9	11.11	8.5	5.57

* 1 mc. of sodium acetate-2- ^{14}C (SA 0.74 mc./m-mole).

† Specific activity = $\frac{\text{Dis./min.}}{\text{m-mole}} \div 1000$.

‡ Percentage derived from substrate = $\frac{\text{SA CO}_2 \text{ or CH}_4}{\text{SA acetate}} \times 100$.

§ Each value mean of three or more observations.

direct cleavage of acetate, the intact methyl group of this substrate being incorporated into methane in accordance with the findings of Buswell & Sollo (1948), Stadtman & Barker (1949) and Pine & Barker (1956).

Calculation of the relative isotope concentration in acetate as precursor and carbon dioxide as product indicates that acetate participated in reactions which led more readily to the formation of carbon dioxide than to the formation of methane. In trial 1 a maximum of 6.5% and in trial 2 a maximum of 11% of the carbon dioxide was derived from the labelled carbon which originally resided in acetate-2-¹⁴C. (Comparable values for methane were 3.8 and 5.6% for trials 1 and 2, respectively.)

Whilst the specific activities of carbon dioxide and methane in trial 2 were rather erratic, in general, the relationship of the specific activity curves of carbon dioxide-¹⁴C and methane-¹⁴C in trials 1 and 2 is that of two products derived from a single precursor. Should there be a decrease in carbon dioxide-¹⁴C in such a manner that its curve crosses that of methane, or an increase in the specific activity of methane so that its curve crosses or touches that of carbon dioxide, then the relationship between the two would be that of precursor (carbon dioxide) to product (methane) (Aranoff, 1956). The point where the specific activity curves cross would be the maximum specific activity of the product. The experimental time period was not extended long enough to determine this final relationship. However, considering the trends of the data, the curves which start as two products probably would end in a new precursor-product relationship.

Table 3 summarizes an experiment in which 1 mc. of acetate-2-¹⁴C (SA 7.5 mc./m-mole) was added to the rumen 30 min. after feeding to determine the effect of maximum rate of rumen fermentation on the incorporation of methyl carbon from acetate into other products of rumen digestion. These data show that the specific activity of acetic acid decreased rapidly during this period as a result of the increased production of acetate in the rumen fermentation. In contrast to the previous trials, the labelling of propionic and butyric acid was more pronounced and the higher volatile fatty acids (greater than C₄) were labelled throughout the experimental period. The specific activity of butyric acid decreased during the first 2 hr., increased during the next 2 hr., and then decreased during the remainder of the trial. The concentration of propionic acid-¹⁴C increased throughout most of the experiment and began to decrease only at the end of the sampling time. The methyl carbon of acetate could account for a maximum of 3.2% of the methane and a maximum of 4.2% of the carbon dioxide. While the % of methane derived from the methyl group of acetate was in the general range of values obtained in the previous experiments, the % of carbon dioxide-¹⁴C was much smaller because of the increased production of carbon dioxide from other sources during this period of maximum activity of the rumen microbiota.

The results from the preceding experiments relative to the contribution of methyl-labelled acetate to rumen methanogenesis and carbon dioxide production suggested an experiment to determine the contribution of the carboxyl group of acetate to the formation of these two gaseous products of rumen digestion. The experimental conditions used were as before except that 2 mc. of acetate-1-¹⁴C (SA 2.25 mc./m-mole) were added to the rumen 18 hr. after feeding, as in the first trial.

Table 3. *Distribution of ^{14}C after addition* of sodium acetate-2- ^{14}C to the rumen $\frac{1}{2}$ hr. after feeding*

Time after addition of ¹⁴ C (hr.)	Volatile fatty acids						Gases					
	Acetate		Propionate		Butyrate		V.F.A. > C ₄		CO ₂		CH ₄	
	(SA)†	(m-mole/ 100 ml.)	(SA)	(m-mole/ 100 ml.)	(SA)	(m-mole/ 100 ml.)	(SA)	(m-mole/ 100 ml.)	(SA)	(%)†	(SA)	(%)
$\frac{1}{2}$	5620§	6.68	0	1.58	736	1.36	247	0.20	3.2	0.06	2.5	0.05
1	1060	6.30	35	1.81	580	1.52	237	0.26	2.1	0.29	3.4	0.33
2	643	6.75	38	1.72	504	1.27	372	0.26	3.6	0.56	8.6	1.34
3	543	7.87	58	1.67	649	1.42	362	0.35	7.2	1.33	6.3	1.17
4	481	5.98	69	2.08	716	1.51	438	0.30	8.7	1.81	5.4	1.13
5	343	7.73	67	2.14	597	1.47	374	0.29	9.4	2.75	5.6	1.65
6	271	7.88	78	1.83	537	1.44	269	0.26	9.4	3.50	6.9	2.54
7	215	8.15	65	2.02	434	1.44	335	0.28	8.9	4.16	7.0	3.24

* 1 mc. of sodium acetate-2-¹⁴C (SA 7.5 mc./m-mole).
† Specific activity = $\frac{\text{Dis./min.}}{\text{m-mole}} \div 1000$.

* 1 mc. of sodium acetate-2- ^{14}C (SA 7.5 mc./m-mole).† Derived from acetate = $\frac{\text{SA } \text{CO}_2 \text{ or } \text{CH}_4}{\text{SA acetate}} \times 100$.‡ Specific activity = $\frac{\text{Dis./min.}}{\text{m-mole}} \div 1000$.

§ Each value mean of three or more observations.

Table 4. *Distribution of ^{14}C from sodium acetate-1- ^{14}C added* to the rumen 18 hr. after feeding*

Time after addition of ¹⁴ C (hr.)	Volatile fatty acids						Gases					
	Acetate		Propionate		Butyrate		V.F.A. > C ₄		CO ₂		CH ₄	
	(SA)†	(m-mole/ 100 ml.)	(SA)	(m-mole/ 100 ml.)	(SA)	(m-mole/ 100 ml.)	(SA)	(m-mole/ 100 ml.)	(SA)	(%)‡	(SA)	(%)
$\frac{1}{2}$	8757§	2.74	0	0.58	59	0.54	0	0.19	1	0.01	5.6	0.06
1	1866	1.60	0	0.60	212	0.66	0	0.05	17	0.92	0.4	0.02
2	1168	3.19	67	0.69	281	0.58	129	0.23	26	2.20	1.2	0.11
3	1035	2.69	79	0.48	277	0.54	0	0.21	39	3.76	1.9	0.18
4	941	3.35	85	0.69	335	0.58	291	0.25	51	5.36	8.8	0.94
5	795	2.86	96	0.67	464	0.49	211	0.22	70	8.85	9.3	1.18
6	640	2.72	108	0.57	392	0.44	0	0.24	64	10.09	9.1	1.43
8	595	2.63	116	0.51	247	0.40	0	0.29	56	9.49	11.7	1.97

* 2 mc. of sodium acetate-1-¹⁴C (SA 2.25 mc./m-mole).
† Specific activity = $\frac{\text{Dis./min.}}{\text{m-mole}} \div 1000$.

* 2 mc. of sodium acetate-1- ^{14}C (SA 2.25 mc./m-mole).† Specific activity = $\frac{\text{Dis./min.}}{\text{m-mole}} \div 1000$.‡ % = $\frac{\text{SA } \text{CO}_2 \text{ or } \text{CH}_4}{\text{SA acetate}} \times 100$.

§ Each value mean of three or more observations.

The results (summarized in Table 4) show that the carbon dioxide was much richer in ^{14}C than the methane. The data also show other differences from the preceding experiments, in that the labelling of butyric acid continued for 5 hr. instead of 3 hr., and the specific activities were higher, even when corrected for the addition of twice as much isotope. The fatty acids with chain lengths greater than C_4 also became labelled in some instances. The propionic acid labelling pattern was similar to that of the first experiment. The differences in the labelling of the various volatile fatty acids suggests that the method of incorporation of carboxyl carbon differs from that of the methyl carbon of acetate. It was calculated that the carboxyl group of acetate- ^{14}C gave rise to a maximum of 2% of the methane and 10% of the carbon dioxide encountered during the experimental time period.

DISCUSSION

Although bacteria which convert acetate to methane and carbon dioxide are readily established in enrichment cultures of bovine rumen contents, the data presented in this report demonstrate that only a small portion of rumen methane was derived from acetate and suggest that these organisms were present in small numbers. This observation is in accord with the report (Nelson, Brown & Kingwill, 1960) that the addition of *d*-limonene to the rumen *in vivo* in concentrations which were toxic to acetate-utilizing methane bacteria *in vitro* (Crane, Nelson & Brown, 1957) did not appreciably alter the ratio $\text{CO}_2:\text{CH}_4$ in rumen gas. Beijer (1952) previously had observed that methane was not formed from acetate by rumen contents *in vitro* during short term incubation.

The active participation of acetate-1- ^{14}C and acetate-2- ^{14}C in reactions leading to the labelling of other higher volatile fatty acids in the rumen pool in these experiments is in agreement with the observations of Gray, Pilgrim, Rodda & Weller (1952) who reported that most of the derived radioactivity was found in the butyrate fraction of rumen fluid after the addition of acetate-1- ^{14}C to the rumen of a sheep. The data presented here extend these observations to include the participation of acetate not only in volatile fatty acid synthesis but also in methanogenesis and in the formation of carbon dioxide in the rumen.

The data demonstrating the participation of acetate-2- ^{14}C in reactions leading to the *in vivo* labelling of other volatile fatty acids and carbon dioxide in the rumen are in direct contrast to the *in vitro* fermentation of this substrate. When the acetate-2- ^{14}C used in the *in vivo* trials was fermented *in vitro* by acetate-stabilized rumen enrichment cultures, essentially all of the radioactivity of the gas resided in the methane and only trace quantities of ^{14}C were found in the carbon dioxide.

It is evident that the participation of acetate in various metabolic reactions in the rumen forestalls any precise measurement of the direct incorporation of the intact methyl group of acetate-2- ^{14}C into methane. However, it is possible to ascribe maximum values for this reaction in terms of the experimental values. Calculation of the relative isotope concentration indicates that a maximum of 3.8 and 5.6% of the methane might have arisen from the intact methyl group. Since the carboxyl carbon of acetate-1- ^{14}C gave rise to 2% of the methane and 10% of the carbon dioxide, it can be postulated that the acetate molecule contributed from 5.8 to 7.6% of the rumen methane and from 16 to 21% of the carbon dioxide generated

in the rumen during the fasting experimental period. The contribution of acetate-2-¹⁴C to carbon dioxide and methane formation during the period of maximum rumen activity immediately after the ingestion of a ration is somewhat less than the above range of values.

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Yeasts from the Bovine Rumen

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SUMMARY

Yeasts belonging to the genera *Candida* and *Trichosporon* have been isolated in small numbers from the rumen of fistulated cows. Similar yeasts were not found on samples of feed material. The yeasts present fell into two groups dependent on the diet of the cow from which they were isolated.

INTRODUCTION

The yeast flora of the lower intestinal tract of cattle was studied by Parle (1957) and by Van Uden & do Carmo Sousa (1957); but in spite of the similarity of the rumen environment to that of the lower intestinal tract, rumen yeasts have received little attention. A rumen yeast flora has not been reported previously although the work of several authors has suggested its existence. Ingram & McGaughey (1948) isolated 'Candida-like' organisms from the rumen of sheep and Cunningham & Brisson (1955) noted an alcoholic fermentation in the stomachs of lambs on a high glucose diet. Lubinsky (1957, 1958) listed yeasts as food for many species of ciliate protozoa from the rumens of reindeer, sheep and goats. Rumen yeasts may not play an important part in the rumen fermentation, but from the veterinary point of view they may be of importance as a reservoir of infection. Yeast infections of the bovine udder are relatively frequent and have been caused by species of *Candida*, *Trichosporon* and *Pichia*. The present investigation into the rumen yeast flora was prompted by the isolation of *Candida albicans* from the rumen during a preliminary survey (Clarke, 1960).

METHODS

The rumen contents of five cows, one normal (no. 34) and four with rumen fistulas (no. 1, 90, 293, 294) were sampled at intervals between January 1959 and February 1960. All cows were fed hay during the winter months (April-August). During the spring and summer (September-February) all cows, with the exception of cow no. 1 which was hay-fed, were given fresh red clover (*Trifolium pratense* L.). As stall feeding could be carried out only on working days (Monday to Friday inclusive), the cows were turned out to pasture on Saturdays and Sundays. In spring and summer the pasture was predominantly red clover and in winter predominantly ryegrass (*Lolium perenne* L.).

Samples of rumen contents were removed from fistulated cows by an operator who wore sterile rubber gloves. The non-fistulated cow was sampled by using a sterile oesophageal tube, with suction.

The pasture, fresh clover and clover hay on which the cows were feeding were each sampled on two occasions. Hay samples were taken with aseptic precautions and clover and pasture samples were cut with sterile box shears.

All samples were plated on glucose peptone agar (4%, w/v, glucose; 1%, w/v, peptone; 2%, w/v, agar; adjusted to pH 4). Serial dilutions of the samples to be plated were made by the method of di Menna (1957). All cultures of rumen samples were incubated at 39°, but in addition two from hay-fed cows and parallel cultures from the non-fistulated cow were incubated at room temperature. Cultures of hay and pasture samples were incubated both at room temperature and at 39°. After 3–6 days of incubation all yeast-like colonies were picked off for identification from plates where the colonies were well separated.

Yeast cultures were maintained on the following medium (% w/v): Malt Extract (Difco) 0.3; Yeast Extract (Difco) 0.3; Neopeptone (Difco) 0.2; glucose 1; in tap water. The yeasts obtained were identified by the criteria of Lodder & Kreger-van Rij (1952).

RESULTS

Rumen isolates

Particulars of the rumen samples and of the yeasts isolated are given in Table 1. Nine species belonging to three genera were identified from the 134 isolates examined. Viable counts made during the isolation procedure ranged from 80 to 13,000 organisms/g. rumen contents. Particulars of the species isolated are given below.

Candida spp. Ten isolates of *Candida albicans* (Robin) Berkhout were recovered from two clover-fed cows and one from a hay-fed cow. Two of the isolates were tested for pathogenicity by intravenous inoculation into rabbits. Both killed rabbits within 48 hr. and were recoverable from the animals' kidneys. Four isolates of *C. tropicalis* (Cast.) Berkhout, four of *C. krusei* (Cast.) Berkhout and two of *C. rugosa* (Anderson) Diddens & Lodder were recovered from two clover-fed cows. One isolate of *C. rugosa* was obtained from a hay-fed cow.

Trichosporon spp. Sixteen isolates of *Trichosporon cutaneum* (de Beurm., Gougerot & Vaucher) Ota were recovered from two cows, both fed on hay. These isolates resembled those of *T. cutaneum* type II described by Lodder & Kreger-van Rij (1952, p. 624) in that cultures on solid media were cream, dull, dry, becoming folded, but not hairy. They differed in that assimilation of maltose was not apparent on solid carbon-source auxanograms and was extremely weak in liquid carbon-source auxanograms. Cultures of two of these isolates were sent to Dr N. J. W. Kreger-van Rij (Yeast Division, Centraalbureau voor Schimmelcultures) who considered them to be variants of *T. cutaneum*.

Eighty-four isolations of *Trichosporon sericeum* (Stautz) Diddens & Lodder were made from four cows, seventeen from a clover-fed cow and the rest from hay-fed cows. Twenty-six of the isolates were recovered from room temperature cultures. In the key to the genus *Trichosporon* Lodder & Kreger-van Rij (1952) separate *T. sericeum* from *T. capitatum* Diddens & Lodder by its reduced mycelium and relatively strong assimilation of galactose. The eighty-four strains isolated here produced mycelium readily under aerobic conditions but also assimilated galactose

Table 1. Yeasts isolated from the bovine rumen

Tempera- ture of isolation (°C)	Cow (no.)	Date	Organisms*								
			C.	C.	C.	T.	T.	R.	R.		
			<i>albicans</i>	<i>tropicalis</i>	<i>krusei</i>	<i>rugosa</i>	<i>cutaneum</i>	<i>sericeum</i>	<i>glutinis</i>	<i>R. muc-</i> <i>laginosa</i>	<i>R. mucra-</i> <i>macrans</i>
			No. of isolates								
39	294	13. i. 59	.	.	1	2
39	294	12. ii. 59	5
39	294	16. ii. 59	4
39	294	17. ix. 59	7	.	.
39	294	28. x. 59	8	.	.
39	294	26. i. 60	.	2	1	.	.	.	2	.	.
39	293	16. ii. 59	.	2	2
39	293	1. ii. 60	1
39	34	27. xi. 59
c. 19	34	27. xi. 59	1	.	2
39	90	15. vii. 59	5	.	.
39	294	19. v. 59	.	.	.	1	6	3	.	.	.
39	293	29. v. 59	1
39	293	3. vii. 59	27	.	.	.
c. 19	293	3. vii. 59	2	26	2	3	.
39	1	1. ii. 60	1	6	.	.	1
c. 19	1	1. ii. 60	3	.	.
Total			11	4	4	3	16	84	6	3	3

* C=Candida; T=Trichosporon; R=Rhodotorula.

strongly on solid auxanographic medium. Authentic strains of *T. sericeum* and *T. capitatum* were obtained from the Centraalbureau voor Schimmelcultures and compared with the bovine isolates. As the results from this comparison were inconclusive, it was decided to allocate the bovine isolates to the longer established species, *T. sericeum*.

Rhodotorula spp. *Rhodotorula glutinis* (Fres.) Harrison, *R. mucilaginosa* (Jorg.) Harrison and *R. macerans* Frederikson were isolated from three cows. All these isolations were made at room temperature. *R. glutinis* and *R. macerans* were the only yeasts isolated from the non-fistulated cow (34).

Hay and pasture isolates

Six species belonging to three genera were identified from the twenty-one isolates obtained from hay and pasture samples. All the yeasts recovered from the hay and pasture samples were from cultures incubated at room temperature; none could be isolated at 39°. No yeasts were recovered from the clover samples. Particulars of the species isolated are given below.

Cryptococcus laurentii (Kufferath) Skinner and *C. luteolus* (Saito) Skinner were recovered from pasture samples.

Torulopsis ingeniosa di Menna was recovered from pasture. *Rhodotorula glutinis* (Fres.) Harrison was recovered from pasture and both *R. macerans* Frederikson and *R. mucilaginosa* (Jorg.) Harrison were found on hay.

DISCUSSION

In view of the similarity of the anaerobic fermentations in the bovine rumen and lower intestinal tract, the presence of yeasts in the rumen is not surprising. With the exception of *Candida albicans*, those yeasts isolated here from cultures incubated at 39° are represented among those found in the bovine caecum by Van Uden & do Carmo Sousa (1957), and fall into their second group of yeasts: facultative saprophytes of warm-blooded animals. It is evident that the yeasts isolated here are true rumen inhabitants since yeasts which could grow at 39° were not isolated from the samples of feed material, and the rumen yeasts were not represented among those considered as part of the normal flora of the leaves of pasture plants (di Menna, 1959). The yeasts isolated here from hay and pasture samples were similar to those found by di Menna (1959). Of these, only *Rhodotorula* spp. were isolated from the rumen, and then only at room temperature.

The yeasts isolated from the rumen at 39° fall into two groups, fermenting and non-fermenting yeasts. The presence of either group appears to be dependent upon the diet of the cows. Except for one isolate of *Candida albicans* all the yeasts isolated from hay-fed cows were of the non-fermenting type. This isolate of *C. albicans* was made from cow no. 1 only a few days after it was returned to a hay diet. All the other fermenting yeasts were recovered from cows fed on fresh clover. This division into fermenting and non-fermenting yeast flora may be dependent on the soluble sugar content of fresh clover and clover hay. The soluble sugar content of fresh red clover may reach as high as 9% of the weight, calculated on a dry-matter basis, while that of clover hay is usually considerably lower depending on the conditions at the time of harvesting (Dr R. W. Bailey, personal communication).

The failure to isolate yeasts other than *Rhodotorula* species from the non-fistulated cow was probably the result of having to use an oesophageal tube for sampling. The samples collected were always small and from an undetermined part of the rumen and reticulum. From experience with fistulated cows it is known that it is difficult to obtain a representative sample of rumen liquor because of layering of the rumen contents.

The numbers of yeasts found in the rumen are too low for them to be important in the provision of soluble fermentation products although they undoubtedly contribute to the total microbial protein available to the animal.

Our thanks are due to Dr N. J. W. Kreger-van Rij (Yeast Division, Centraal-bureau voor Schimmelcultures, Delft, Netherlands) for her identification of the *Trichosporon cutaneum* cultures.

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The Influence of Environment on Antigen Production by *Pasteurella pestis* Studied by Means of the Continuous Flow Culture Technique

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SUMMARY

The yields of three antigens of *Pasteurella pestis* (fraction I, antigen 4, V antigen) in continuous flow culture over a range of temperature and pH values have been determined quantitatively. The production of some other antigens was followed qualitatively. The production of fraction I, V antigen and antigen 4 was greatest at 37° and little or none at 28°. The production of antigen 4 required an environmental pH below 6.9. The production of antigen 5 showed oxygen dependence at 28° but not at 37°. In transitions from one antigenic state to another the amounts of some antigens in the organism passed through maxima. Selection against types with the V and fraction I antigens occurred during growth at 37°, but not at 28°. Selection against types with antigen 4 did not occur.

INTRODUCTION

To produce organisms of constant antigenic composition, quantitatively and qualitatively, strict control of environment would seem to be necessary. We became interested in this problem because of the need to control the antigenic composition of *Pasteurella pestis* in studies of the immunogenicity and virulence of this organism. Continuous flow culture rather than batch culture was used because of the possibility of maintaining a given environment indefinitely in the continuous system. Consideration of the differences between a batch process and a continuous process leads one to expect that the two techniques may give organisms of different antigenic composition. This may result because, in the course of the batch process, especially one which requires a complex medium (as pathogenic bacteria usually do) there may be environmental changes (in available nutrients or in physical factors such as pH value) unless adequate control equipment is available. Different antigens may be produced at different stages in batch culture; *P. pestis* provides an example of this (Crumpton & Davies, 1956). On the other hand, in a continuous flow culture one can select any environment from that of the log phase to the stationary phase and maintain it indefinitely, but one may then find that, because the organisms grow in only one environment, not all the antigens can be produced at once. The way out of this difficulty should be to use a multi-stage process (Pirt & Callow, 1959; Callow & Pirt, 1961). Data about these problems is provided by the present work. The lack of control over, and the variation of conditions in batch culture may explain why, with rare exceptions, the published work on the antigenic

composition of micro-organisms does not indicate that variation in environment is a reliable way of altering the antigenic composition. The problem of the effect of environment was taken up by Lacey (1953) who found that reversible changes in the antigenic composition of *Haemophilus* spp. could be induced by variation in the proportions of Na, Mg, Cl and SO_4 ions, and in the temperature of the medium. Ogburn, Harris & Harris (1958) used a continuous flow technique to show that an extracellular antigen of a haemolytic streptococcus required an acid pH value for its formation. Striking examples of the effects of environment on antigenic composition have been found with *Paramecium aurelia* (Beale, 1954) where the antigenic composition is a function of the temperature, the inorganic salt composition of the growth medium and the amount of nutrients supplied.

The earliest recognition of the effect of an environmental factor on antigen production by *Pasteurella pestis* seems to have been that of Schütze (1932) who found that the envelope antigen (now known as fraction I) was produced at 37° but not at 26° , although earlier Rowland (1914) had recognized the temperature dependence of envelope formation. For an historical review of the antigenic analysis of *P. pestis* reference should be made to Crumpton & Davies (1956). These authors, by application of gel diffusion analysis, revealed the presence of seven new antigens. One of these antigens was antigen 4, later characterized in greater detail and shown to be of immunogenic importance (Crumpton & Davies, 1957). The requirements for antigen 4 production seemed to be exacting because, although produced in an aerated broth galactose medium at 37° , the antigen did not appear in the organisms until 16 hr. after the initiation of growth. This antigen was also absent from organisms grown as surface cultures on broth agar. An explanation of this behaviour is provided by our results. Further development of knowledge about *P. pestis* antigens came from the recognition by Burrows & Bacon (1956) of the V and W antigens as characteristic of the virulent strains. The present work has three parts: quantification of the effects of pH value and temperature on antigen production; a description of some transitional stages when the antigenic composition is changed by the environment; some effects of variation and selection on antigenic composition in long-term cultures and their control by the environment.

METHODS

For descriptions of methods other than those given below reference should be made to Pirt & Callow (1958). The method of dry wt. determination was used with formalized samples (HCHO concn. 3%, w/v).

Continuous flow-culture technique. The culture apparatus was based on that of Elsworth, Meakin, Pirt & Capell (1956) and Callow & Pirt (1961), but modified to ensure safety in work with these pathogenic organisms. The medium flow rate was 0.1 culture volumes/hr. (dilution rate 0.1 ± 0.005 hr.⁻¹); hence the generation time of the organisms was 6.9 hr. in the steady state.

The pH value was controlled by the automatic addition of 2N-NaOH or 2N- H_2SO_4 (Callow & Pirt, 1956); the pH controller (Fielden Ltd., Paston Road, Wythenshawe, Manchester) controlled within ± 0.15 pH units. Temperature was controlled within $\pm 0.5^\circ$: changes in pH value and in temperature were made gradually at rates of 0.1 pH unit or $1^\circ/4$ hr.

Media. The liquid medium contained (g./l.): casein acid hydrolysate (Oxoid) 15.0; L-cysteine monohydrochloride, 0.32; glycine, 1.5; glucose, 12.0; KH_2PO_4 , 0.85; $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 8.0; trisodium citrate dihydrate, 3.48; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.6; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.03; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.02; CaCl_2 , 0.0224. The final pH value was 7.4. Sterilization was by autoclaving (126° for 20 min.); the glucose and cysteine solutions added were sterilized separately.

Either Alkaterge-C (Commercial Solvents Corp., Terre Haute, Indiana, U.S.A.) 50 % (v/v) in liquid paraffin, or silicone antifoam B (Midland Silicones, Ltd) 25 % (w/v) was added as an antifoam.

The solid medium contained agar with tryptic digest of meat to which 5 % of its volume of Fildes peptic digest of sheep's blood (Mackie & McCartney, 1948) was added.

Organisms. The avirulent Tijwidej smooth (TS) strain of *Pasteurella pestis* and the virulent L37 strains were used. The stock cultures were maintained as freeze-dried cultures or in stab cultures on the solid medium at 4° .

Preparation of inoculum. A slope of the solid medium was inoculated from the stock culture. The slope culture was transferred to a conical flask containing 12.5 ml. liquid medium with the following changes: KH_2PO_4 , 3.2 g./l.; Na_2HPO_4 , 13.6 g./l.; casein hydrolysate 10 g./l.; glucose replaced by galactose, 10 g./l.; glycine and CaCl_2 omitted. These changes were used principally to increase the buffer capacity and to restrict the growth to be more in keeping with the amount of available oxygen. After 24 hr. gentle reciprocal shaking (throw 0.6 in., 100 cycles/min.) the culture was transferred to 100 ml. of the same medium in a 2 l. bottle and shaken gently for 24 hr. when the culture was used to inoculate the continuous flow culture, 2 l. in volume. Initially, to ensure growth, excess aeration had to be avoided; for the first 2 or 3 generations a sulphite oxidation value of 5 mmole O_2 /l./hr. was adequate, with no air flow through the culture vessel.

Antigenic analysis. The organisms were prepared by centrifuging down and drying with acetone at -20° . The supernatant liquid was examined after it had been freed from organisms by membrane filtration.

Qualitative antigenic analysis was carried out by the Ouchterlony agar gel diffusion method as described by Crumpton & Davies (1956). Photographic records were made at about the fourth day. For the detection of V antigen the micro gel diffusion method of Mansi (1958) with 4 mm. between wells was used for economy of antiserum and antigens. The diffusions for qualitative analysis were carried out at ambient temperature (*c.* 22°). For the identification of antigens other than V antigen a standard line pattern obtained with TS organisms and antiserum provided by Dr D. A. L. Davies (this Department) was used. For the detection of V antigen a standard line pattern showing V and W antigens only was obtained with serum and organisms provided by Dr T. W. Burrows (this Department). The organism preparations of Davies & Burrows are referred to as the standard batch culture preparations.

Methods of quantitative antigenic analysis were developed by using the micro gel diffusion method of Mansi (1958) with specific antisera. The method consisted of comparing the highest dilution of the standard batch culture preparation of antigen which would give a line on the plate not coincident with the antigen well, with the highest dilution of the sample which did likewise. The unit of antigen was defined as the amount present in 1 mg. of the standard batch culture preparation; the units

of antigen/mg. sample were calculated from the dilutions. The factor between antigen dilutions was 2. The distance between wells on the plate was 2 mm. Fraction I, antigen 4 and V antigen were estimated in this way. The diffusion temperature for the quantitative assays of fraction I and antigen 4 was 37° for 4 and 3 hr., respectively. The diffusion of the V antigen was at *c.* 22° for 18 hr.

RESULTS

Growth of the organism

The average yield of either strain when there was an excess of available oxygen was 5.5 mg. dry wt./ml. at 28° and 3.7 mg. dry wt./ml. at 37°. This difference between the yields expresses quantitatively the known fact that growth of *Pasteurella pestis* is poorer at 37° than at 28°. The mean total number of organisms/mg. dry wt. was 2.5×10^9 .

With an excess of available oxygen the oxygen uptake rate was 29 mmole/l./hr. at either temperature. All the results with the TS strain were obtained with excess of available oxygen, that is, with respiration unlimited by oxygen supply. In work with the L37 strain the oxygen uptake rate was limited to 9 mmole/l./hr. when it was found that, with one exception noted below, an excess of oxygen was not essential to the production of the antigens in which we were interested. Limitation of the aeration had the advantage of making foam control easier. This restriction of the oxygen supply decreased the dry wt. yield of organism by 35 %.

The yield of organism was independent of the pH value in the range pH 6.3–7.3. The TS strain was grown with undiminished yield at pH 5.9 and 37°. An advantage of using pH control instruments was that glucose could be used as a carbon source. Previous workers, without using this control method, found that acid production from glucose soon stopped growth; this difficulty was previously overcome by using galactose as a carbon source.

Apart from the effect on the yield of organism, increase in the temperature from 28° to 37° also caused a severe shock to the culture. This shock, which was particularly marked in growing cultures supplied with an excess of oxygen, was characterized by a high death-rate of the organisms, the appearance of much cell debris, much foaming, and decreases in dry wt. and oxygen uptake to much below the eventual steady-state values. This shock was largely avoided when growth was limited by the oxygen supply.

Production of antigen 4

The production of antigen 4 was dependent on pH value and temperature. The pH value dependence is illustrated in Fig. 1. Production of antigen 4 was completely suppressed at pH values above 6.9. The optimum pH value for antigen 4 production was pH 5.9, at which value the amount of antigen present in the organisms was six times greater than the amount in the standard batch culture preparation. The supernatant fluid contained a large amount of the antigen, about twice the amount found in the organisms. The dialysed solids of the supernatant fluid at pH 5.9, we are informed by Dr M. J. Crumpton, were about 20 % pure antigen 4, which means that this fluid was the richest source of antigen.

The effect of pH value on antigen 4 production by the L37 strain was the same as with the TS strain. The range of yields of the antigen by the two strains are given in

Table 1. The yields of antigen 4 are compared at pH 6.3 instead of at the optimum pH value of 5.9, because we found it convenient to work in the range pH 6.3–7.3 and thus to decrease the time necessary for the pH changes. The effect of temperature on antigen 4 production is shown in Table 2. At 28°, a small amount of the antigen was produced at pH 6.3; the production was 20 times greater at 37°. At 28° and pH 7.3 antigen 4 was undetectable. Antigen 4 production in the culture was characterized by a marked increase in the sedimentation time of the organisms; this is in agreement with the finding of Crumpton & Davies (1957) that this antigen stabilizes a suspension of the organisms.

Production of fraction I and V antigen

Fraction I production was independent of environmental pH value in the range pH 6.3–7.3, but was dependent on the temperature (Table 3). At 28° only trace

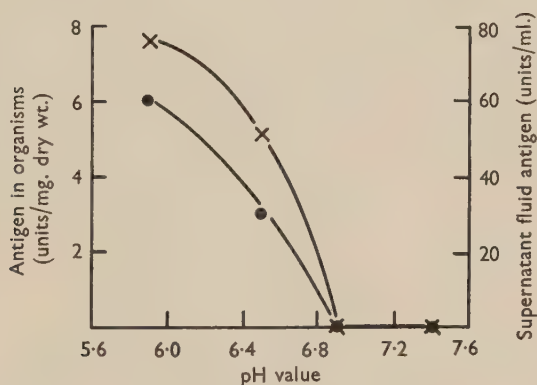


Fig. 1. Antigen 4 production by the TS strain of *Pasteurella pestis* as a function of pH value at 37°. ●, Antigen in organism; x, supernatant fluid antigen. The values were obtained in the same culture. In this experiment the medium was modified in that the concentrations of casein hydrolysate, glycine and glucose were increased by 33%; and CaCl_2 was omitted.

Table 1. Comparison of antigen 4 production by the TS and L37 strains of *Pasteurella pestis* at high and low pH values at 37°

Pooled results from four different cultures of each strain; each set of conditions was reproduced two or more times.

Strain of <i>P. pestis</i>	pH 7.3	pH 6.3
	Antigen in organism (units/mg. dry wt.)	
TS	< 0.06	0.5–2.0
L37	< 0.06	1.0–1.0
	Antigen in supernatant fluid (units/ml.)	
TS	< 1.28	2.6–12.4
L37	< 1.28	2.6–10.2

The very high values for antigen 4 production reported in Fig. 1 are not comparable with those above because of important differences in media used.

Table 2. *Effect of temperature on antigen 4 production at pH 6.3 by Pasteurella pestis*

Strain of <i>P. pestis</i>	Growth temperature	
	28°	37°
	Antigens in organisms (units/mg. dry wt.)	
TS	0.125	2.0
L37	0.03	1.0
	Antigen in supernatant fluid (units/ml.)	
	28°	37°
	TS	2.56
L37	< 1.28	5.1

The values for each strain are from the same culture, those for 28° being obtained before those for 37°

Table 3. *Effect of temperature on fraction I production by Pasteurella pestis*

Strain of <i>P. pestis</i>	Growth temperature	
	28°	37°
	Fraction I in organisms (units/mg. dry wt.)*	
TS	0.0-0.03	0.25-0.50
L37	0.0-0.06	0.125-0.50
	Fraction I in supernatant fluid (units/ml.)*	
TS	0.0-0.03	1.28-2.26
L37	< 0.32	0.32-1.25

* Maximum amount in steady states. Pooled results of four cultures of each strain: each set of conditions was reproduced two or more times.

amounts of the antigen were produced; at 37° the antigen was produced in large amount, though the maximum fraction I content of organisms was only about a half of that of the standard batch culture preparation. About 50 % of the total fraction I antigen content of the culture was present in the supernatant fluid.

V antigen was not detected in organisms grown at 28°. At 37° a maximum V antigen content of 1 unit/mg. dry wt. was obtained; this equals that of the standard batch culture preparation. None of this antigen was detected in the unconcentrated supernatant fluid. The V antigen production was constant over the range pH 6.3-7.3.

Production of other antigens

We also identified in our samples antigens 1, 2, 5 and 8 as defined by Crumpton & Davies (1956). Although the amounts of these antigens varied from time to time, their synthesis, with the exception of antigen 5, could not be correlated with any particular conditions. The W antigens of the virulent organism accompanied the V antigen but, judging by the line intensity in the diffusion analysis, were present in very small amount.

The synthesis of antigen 5 seemed to be oxygen dependent at 28° with the L37 strain (the TS strain was not examined in this respect). Organisms grown at 37° gave a strong antigen 5 line in gel diffusion whether or not growth was limited by oxygen. But at 28° a strong antigen 5 line was only produced with excess of available oxygen. Crumpton & Davies (1956) reported that antigen 5 production was highly dependent on temperature; this would appear to be true only when the growth is oxygen limited.

*Rate of change in antigenic composition with
change in environment*

It is known that after a substrate change there must often be a period of adaptation while the metabolism of the organism adjusts itself to this change. Here we are concerned with the adaptation to a change in the physical environment such as that of temperature or pH value. There also arose the practical question of how quickly could a change in temperature or pH value be made without adverse effects on the organism. Generally we found that rates of change in temperature of 1° and of pH value of 0.1 unit, every 4 hr., were tolerated.

In the following discussion it may be borne in mind that, in a continuous culture in a steady state, the rate of antigen production per unit weight of organisms (q_{ag}) may be derived from the antigen concentration by the equation which relates rate of product formation with product concentration:

$$q_{ag} = \frac{\text{antigen concn.} \times D}{x},$$

where D is the dilution rate and x the concentration of organism. Hence, if D and x are constant, q_{ag} is directly proportional to the antigen concentration.

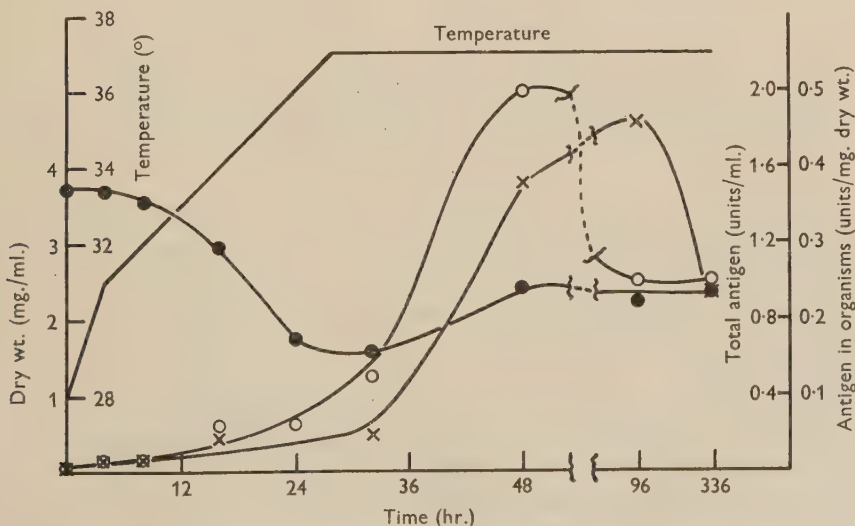


Fig. 2. Fraction I antigen production by strain L37 of *Pasteurella pestis* as a function of time on raising the temperature from 28° to 37°. O, Antigen in organism; ×, total antigen (supernatant fluid + organism); ●, dry wt. of organisms (growth was oxygen limited).

Changes in the fraction I content and in the dry wt. of organism as functions of time on raising the temperature from 28° to 37° are shown in Fig. 2. The main features of the transition are that the dry wt. quickly responded to the temperature change and passed through a minimum before it adjusted itself to the new steady-state value. The production of antigen fraction I, however, did not respond as rapidly as the dry wt. but increased rapidly only when the temperature reached 37°. The amount of fraction I antigen in the organisms passed through a maximum. This may be attributed to an initial retardation of diffusion of the antigen from the organism into the supernatant fluid; it was not due to a decrease in the rate of antigen synthesis since the total amount of antigen present increased when the organism antigen content decreased. The fall in total antigen observed between 96 and 336 hr. may be attributed to selection of fraction I—organisms, that is, organisms without the ability to produce fraction I.

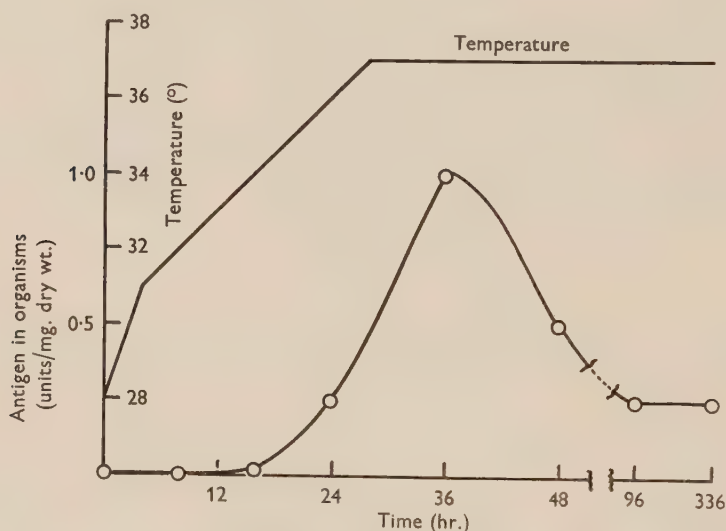


Fig. 3. V antigen production by strain L37 of *Pasteurella pestis* as a function of time on raising the temperature from 28° to 37°.

The transition to V antigen production is shown in Fig. 3. When the temperature exceeded 34° the production of V antigen rose rapidly (in 4 generation times) from zero to maximum. Unlike antigen fraction I, V antigen was not detected in the supernatant fluid. To account for the peak value in the V antigen content it seems necessary to postulate that the initial rate of antigen production exceeded the steady-state rate.

The transition to antigen 4 production is shown in Fig. 4. The transition began when the pH value fell below 6.9. With decrease in pH value the antigen production rate rose rapidly to the maximum. Again, the results show that in the transition the antigen content of the organisms passed through a maximum in a way similar to the transition to fraction I antigen production. The reversibility of the transition to antigen 4 production is illustrated in Fig. 4. On raising the pH value from 6.3 to 7.2 antigen 4 production stopped and the residual antigen was diluted out.

*Maintenance of antigen synthetic abilities;
variation and selection*

An organism, which, given the right environment, is able to produce fraction I antigen is termed a fraction I+ organism; an organism which has lost this ability is termed a fraction I- organism. The terms V+ organism and V- organism are derived similarly.

During cultivation at 37° the organisms gradually lost their abilities to produce both fraction I and V antigens as shown in Tables 4 and 5. These changes we

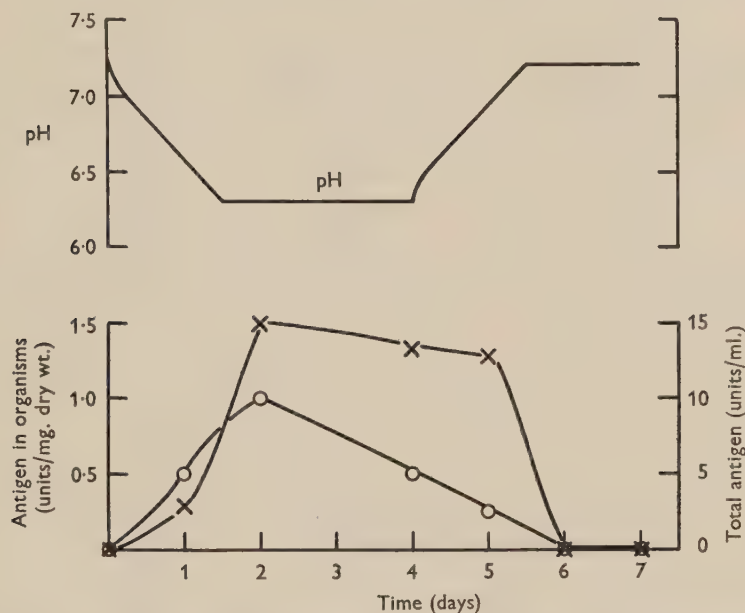


Fig. 4. Antigen 4 production by strain TS of *Pasteurella pestis* as a function of time with pH changes. O, antigen in organism; x, total antigen (supernatant fluid+organism). The dry wt. concn. was 2.6 mg./ml. (in this medium the casein hydrolysate, glycine and glucose concentrations were decreased by one-third).

attribute to selection against fraction I+ and V+ organisms. In the L37 culture (Table 4) there was a 75% decrease in the concentration of fraction I antigen, and consequently in the rate of production, in 59 generations (69th to 128th). Selection against V+ organisms was slower than that against fraction I+ organisms.

At 28° selection against fraction I+ and V+ organisms did not occur. It was possible to grow the organism for any length of time (at least up to 111 generations) at 28° without affecting the ability to produce fraction I antigen when the temperature was raised to 37°. Ability to produce V antigen was unaffected by cultivation of the organism at 28° for at least 55 generations.

The antigen 4 production by the culture over a long period is shown in Table 5, which shows that there was no significant loss in the ability to produce antigen 4 after 210 generations. Hence antigen 4 production may be classed as a stable function of the culture under the conditions used.

Practically all the antigen synthetic functions other than those for fraction I and V antigens seemed to be stable over long periods under the conditions used, judging by the qualitative analyses of line patterns in gel diffusion. In TS cultures after about 150 generations at different pH values and temperatures, ten antigens of the original twelve were detected; fraction I antigen was one of the missing components. Similarly, in L37 cultures, thirteen antigens of the original sixteen were retained, fraction I and V antigens being two of the missing components.

Table 4. *Maintenance of antigen synthetic abilities over long periods by strain L37 of Pasteurella pestis*

Culture age (hr.)	No. of generations	Steady-state concentrations	
		Fraction I antigen in organisms (units/mg. dry wt.)	V antigen in organisms (units/mg. dry wt.)
455	69	0.25	0.25
695	104	0.25	0.25
790	118	0.125	0.50
862	128	0.062	0.25
1462	215	< 0.007	0.125

The temperature was 37° from the 55th to the 128th generation, and from the 166th to the 215th; otherwise it was 28°.

Table 5. *Maintenance of antigen synthetic abilities over long periods by strain TS of Pasteurella pestis*

Culture age (hr.)	No. of generations	Steady-state concentrations	
		Fraction I antigen in organisms (units/mg. dry wt.)	Antigen 4 in organisms (units/mg. dry wt.)
214	33.5	0.25	N.R.
238	36.9	0.25	N.R.
286	44	0.06	0.50
310	47	0.06	N.R.
363	55	< 0.007	N.R.
622	92	< 0.007	2.0
646	96	< 0.007	1.0
670	99	< 0.007	2.0
1030	151	< 0.007	0.5
1438	210	< 0.007	0.5

The temperature was 37° from the 16th to the 55th generation, from the 89th to the 151st, and from the 197th to the 210th; otherwise it was 28°.

The pH value of the culture was 6.3 when antigen 4 production was determined and either pH 6.3 or 7.3 when fraction I and V antigens were determined.

N.R. means no result.

DISCUSSION

Conditions for producing various antigens. A pH value below 6.9 seems essential for the production of antigen 4, this fact and the pH-time curves for growth in batch cultures (unpublished work of Ross, Hakes & Herbert of this Laboratory) explain the apparently exacting requirements for antigen 4 production which Crumpton & Davies (1956) observed. When galactose is the carbon source it takes about 16 hr. for the pH value to fall below 6.9 from the initial value 7.5; this

accounts for the time lag before the appearance of antigen 4 in the cultures of Crumpton & Davies. The absence of antigen 4 from broth agar cultures may be attributed to the absence of galactose or glucose from the medium since, in the absence of the sugar, there is no acid production and the pH value remains too high for antigen 4 production.

The identity between the conditions necessary for the change to antigen 4 production, and those which Aronson & Bichowsky-Slomnicki (1960) found to cause a marked lowering of the electrophoretic mobility of *P. pestis*, suggests that antigen 4 may be the surface component postulated by Aronson & Bichowsky-Slomnicki to explain the change in electrophoretic mobility. However, one piece of evidence seems at variance with this interpretation, namely that the change in electrophoretic mobility was observed to occur not only with the TS, L37 and other strains producing antigen 4, but also with the TRU strain which Crumpton & Davies (1956) classified as a non-producer of antigen 4. This discrepancy may be explained by the presence in the TRU organism of a substance derived from antigen 4 by a slight molecular change sufficient to destroy the antigen specificity but not sufficient to change the electrophoretic properties of the molecule.

The results of Crumpton & Davies (1957) show that antigen 4 is important in the production of immunity to *Pasteurella pestis* by the live TS strain in mice. If production of immunity in this way involves some *in vivo* production of antigen 4 it must mean that the organism finds some environment *in vivo* where the pH value is less than 6.9, that is, considerably lower than the so-called 'physiological' value, pH 7.4.

The qualitative differences in antigenic composition which can be induced by environmental changes make it appear that this is a technique which deserves to be used more in antigenic analysis in the same way as genotypic variants are used. Clearly we have not studied all the environmental changes of interest. In particular it would be of value to study the effect of inorganic salt changes in view of their effects on the antigenic composition of *Haemophilus* (Lacey, 1953) and *Paramecium* (Beale, 1954). Variation in antisera to organisms grown in differently controlled environments is another aspect which requires study, for example, to see whether new antigens are produced.

Transition from one antigenic state to another. It is important to notice that the highest concentrations of some antigens may arise not in any steady states but in the transition from one steady state to another. Practical use might be made of this finding to produce the highest possible yields of some antigens. It may also help to account for the differences between the antigenic composition of organisms grown in batch culture and that of organisms grown in a continuous steady-state culture, since transitional states may occur in batch culture.

Evolution in continuous flow cultures. In long-term cultures (the longest used here was 2300 hr.; 330 generation times) such as we have been studying, selection of variants is a problem to be reckoned with. It has been previously reported (Taylor, 1933) that *Pasteurella pestis* loses virulence more rapidly by *in vitro* culture at 37° than at 27°. An explanation of this is provided by the observation of Burrows (1960) that V- and W- organisms have a selective advantage over their V+ and W+ counterparts, when mixed cultures are incubated at 37°, though not at 28°. Our observations on selection in continuous flow cultures confirm the observations of

Burrows and, furthermore, show that fraction I— organisms have a selective advantage over the fraction I+ organisms at 37°; this gives an added reason why virulence is lost during *in vitro* culture at 37°. If it be desired both to preserve the abilities to synthesize V and fraction I antigens and to produce these antigens in a continuous flow process, it would seem to require more than one stage. The organisms would have to be grown at 28° to prevent selection against V+ and fraction I+ organisms and then transferred to a second stage at a higher temperature for the production of the desired antigens.

We thank Drs D. A. L. Davies, M. J. Crumpton and T. W. Burrows for initiating us into antigenic analysis of *Pasteurella pestis*, and for the supply of standard antigen preparations and antisera.

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The Effect of Formaldehyde on the Shape of Bacterial Flagella

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SUMMARY

Addition of formalin (40 % formaldehyde) to a concentration of 5-10 % (v/v) to a bacterial culture before flagellar staining may change the shape of the flagella as seen in the stained preparation. With many bacteria, if not a majority, the formalin has no effect on the flagellar shape. The coiled, the straight, the small-amplitude and the typical undulant flagellar shapes evidently are mainly due to formalin fixation. The effect of formalin on flagellar shape may have taxonomic significance and should be recorded. Formalin fixation is advantageous in flagellar staining in that it kills the bacteria and makes for nicer preparations, especially when the bacteria are capsulated.

INTRODUCTION

For the past decade the author has routinely added 5-10 % (v/v) of formalin (40 % formaldehyde solution) to all bacterial cultures before washing and flagellar staining. The original reason for this was to kill the bacteria and lessen the danger when working with pathogenic cultures. At the time the formalin procedure was adopted the flagella on the bacteria under study did not show any difference in appearance as a result of the formalin treatment. The consequent assumption that formalin had no effect on flagellar shape will here be shown to be incorrect. In his *Atlas of Bacterial Flagellation* (Leifson, 1960) the author described the various flagellar shapes which may be observed in stained preparations of formalin-fixed cultures. The most common shape observed is that of a fairly uniform wave with a wavelength: amplitude ratio of about 4:1. The author labelled this the 'normal' shape. From published darkfield observations such as those of Pijper & Abraham (1954) and from electronmicrographic studies (Stocker, 1956) it is evident that the usual shape of bacterial flagella is helical. When the flagella dry on the glass surface the helix becomes flattened and the familiar wave shape results. In addition to the normal flagella many bacteria, and most peritrichously flagellate types, may have flagella of wavelength about one half that of the normal flagella. The author has labelled this the 'curly' type. The curly type has also been observed by Pijper in his studies of living bacteria. With many bacteria the normal \rightarrow curly variation appears to have a genetic basis; in others a change of pH value can cause the change of wavelength. Aside from the normal and curly flagellar shapes the various other recorded shapes such as 'coiled', 'straight', 'small amplitude' and 'undulant' would appear to be mainly a result of formalin fixation.

Formalin-fixed cultures of several types of bacteria show flagella of the coiled shape. Most *Serratia* strains show normal flagella in unfixed preparations and coiled flagella in formalin-fixed preparations (Pl. 1, figs. 1, 2). A smaller proportion show curly flagella in unfixed preparations and coiled flagella in formalin-fixed preparations (Pl. 1, figs. 3, 4). The only exception ever observed is a strain of *Serratia indica* (NCIB 2847) with small amplitude and straight flagella in unfixed preparations, and normal flagella in formalin-fixed preparations (Pl. 1, figs. 5, 6). A strain of *Listeria* (B-3D) showed coiled flagella in formalin-fixed preparations and normal flagella in unfixed preparations. The same is true for a culture originally labelled *Aeromonas liquefaciens* (Kluyver L-418) as illustrated in Pl. 1, figs. 9 and 10. A few strains of polar multitrichous pseudomonads have shown coiled flagella in formalin-fixed preparations and normal flagella in unfixed preparations. The flagella of some bacteria may show some coiling in unfixed preparations, but the coiling is invariably enhanced by formalin fixation.

Among peritrichous flagellated bacteria formalin-fixation only rarely produces the straight or small amplitude type of flagella. An example of this is shown in Pl. 1, figs. 7 and 8 with *Listeria* (B-3A). Incidentally it might be worth noting that this strain of *Listeria* and one other showing small amplitude flagella are practically non-motile, which indicates some flagellar abnormality. However, bacteria with typical normal flagella after formalin fixation but without apparent motility also exist. Among the polarly flagellate bacteria straight and stiff flagella have occasionally been observed following formalin fixation. Partially straight or undulant flagella are more frequently observed following formalin fixation, particularly among the marine pseudomonads (Pl. 1, figs. 11, 12). These undulant flagella which result from the formalin treatment must not be confused with flagella of long wavelength and large amplitude often found on polarly flagellate bacteria and which are normal for these bacteria.

DISCUSSION

For the sake of clarity a description of flagellar shape should always include a statement about the method of fixation, whether with formalin or other chemical or none. Preferably both the unfixed and the fixed shapes should be recorded. Formalin fixation is of considerable value in flagellar staining: pathogens are killed; flagella of slime-producing and capsulated bacteria are more readily stained after formalin fixation. The latter is particularly striking with halophilic marine bacteria. The differences in the effect of formalin on the shape of bacterial flagella may have taxonomic significance and the terms 'formalin sensitive' and 'formalin insensitive' may be suggested.

When the flagellar shape is normal or curly after formalin fixation the probability is very small that unfixed preparations will show a different shape. When the flagellar shape is coiled, straight, small amplitude or typically undulant after formalin fixation, the probability is great that the unfixed preparation will show flagella of normal or curly shape.



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EXPLANATION OF PLATE

Effect of formalin fixation on the shape of the flagella of *Serratia* and three other types of bacteria. Leifson flagella stain, $\times 2000$.

Figs. 1, 2. *Serratia kielensis* (NCIB 4619). In fig. 1 is shown the unfixed preparation with normal flagella and in fig. 2 the formalin fixed preparation with coiled flagella.

Figs. 3, 4. *Serratia marcescens* (Smith). In fig. 3 is shown the unfixed preparation with curly flagella. In fig. 4 is shown the formalin fixed preparation with coiled flagella and one curly flagellum.

Figs. 5, 6. *Serratia indica* (NCIB 2847). In fig. 5 is shown the unfixed preparation with small amplitude and straight flagella and in fig. 6 the formalin fixed preparation with normal flagella. The flagellar shape of this strain of *Serratia* is unique in the author's experience.

Figs. 7, 8. *Listeria monocytogenes* (B-3A). In fig. 7 is shown the unfixed preparation and in fig. 8 the formalin fixed preparation. The flagella become straight and stiff as a result of the formalin fixation.

Figs. 9, 10. *Aeromonas (Aerobacter) liquefaciens* (Kluyver L-418). In fig. 9 is shown the unfixed normal flagella and in fig. 10 the formalin fixed coiled flagella.

Figs. 11, 12. *Pseudomonas* sp. (halophilic marine type, Colwell). In fig. 11 is shown the unfixed normal flagellum and in fig. 12 the formalin fixed undulant flagella. This is typical of many but not all of marine pseudomonads.

The Use of the Thionin Blue Sensitivity Test in the Examination of *Brucella*

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(Received 24 November 1960)

SUMMARY

A total of 171 cultures of *Brucella*, mostly isolated in this country from milk and foetal material, was examined for sensitivity to thionin blue as well as to basic fuchsin and thionin, and for agglutination in monospecific sera. Standard strains of *Brucella abortus*, *B. melitensis* and *B. suis* grew in the presence of thionin blue in the concentration studied. Three groups of *B. abortus* were found, however, which were sensitive to thionin blue. These were: (a) strains resembling the vaccine strain *B. abortus* strain 19; (b) dye sensitive strains (*B. abortus* type II Wilson), (c) a previously undescribed group. Cultures of this last group required added CO₂ for growth, grew on basic fuchsin and were virulent for guinea-pigs. The significance of these results is briefly discussed.

INTRODUCTION

McLeod (1944) reported that *Brucella abortus* strain 19 would not grow on media containing thionin blue in concentrations that would allow the growth of other strains of *B. abortus*. This observation was confirmed by Levine & Wilson (1949) and Cruickshank (1954). The latter author also pointed out that strains of *B. abortus* type II Wilson (Huddleson, 1955) did not grow on thionin blue media. These strains are known to be very sensitive to dyes and require the presence of serum or Tween 40 for growth (Huddleson, 1956; Sackman, 1957; Morgan, 1960). The purpose of the present paper is to record the results of the examination of *Brucella* cultures for sensitivity to thionin blue.

METHODS

Organisms. Most of the organisms studied were isolated at this Laboratory from foetal material or milk; the remainder had been sent in for confirmation of identity. Except for recently isolated cultures, they were all checked for dissociation by using obliquely transmitted light (Henry, 1933) and only smooth cultures were studied.

Media. Serum glucose agar was used for the propagation of cultures and as basal medium for the addition of dyes. The composition of this medium has been described (Morgan, 1960).

Basic fuchsin (supplied by Pharmaceutical Laboratories National Aniline Division, Allied Chemical and Dye Corporation, New York) was added to the melted and cooled medium just before pouring plate² to give a concentration of 1/25,000.

Thionin (Allied Chemical and Dye Corp.) was used at a concentration of 1/50,000. Initially, three concentrations of thionin blue (British Drug Houses Ltd., London) were used, namely, 1/500,000, 1/1,000,000, 1/2,000,000. Later however only the 1/500,000 concentration was used for routine use. Stock solutions of the dyes were made and steam sterilized. All new batches were checked for activity against known strains. All plates were incubated overnight at 37° before use and no plate was used which had been stored for longer than one week in the refrigerator. The three F.A.O./W.H.O. reference strains *Brucella abortus* strain 544, *B. melitensis* strain 16M and *B. suis* strain 1330, as well as *B. abortus* strain 19 were always included in each series of tests.

All cultures were tested for the requirement of additional CO₂ (above that present in air) for growth, as soon as possible after isolation. Two slopes were inoculated with each isolate; one was incubated aerobically at 37° and the other in an atmosphere containing 10% (v/v) added CO₂. Slopes were examined after 4 days of incubation.

For the dye sensitivity tests, cultures were incubated for 48 hr. in the CO₂ enriched atmosphere and the growth suspended in buffered saline (pH 6·8) to give a concentration of approximately 3×10^9 organisms/ml. One loopful of the suspension was streaked five times on each of the dye plates, without recharging the loop, using a quarter plate per suspension. The loop was sterilized between each plate. All the plates were incubated in the CO₂ enriched atmosphere and read after 5 days of incubation at 37°. The results for each dye were interpreted as negative or 1+ to 5+ depending on whether growth had occurred on 1 or all 5 streaks.

Serological test. Monospecific sera for *Brucella abortus* and *B. melitensis* were prepared from rabbit sera by absorption with the heterologous antigen. One loopful of the serum, diluted 1/5 in phenol saline, was placed on a slide and the culture emulsified. The suspension had not to be too concentrated, otherwise agglutination was delayed. Occasionally the results of slide agglutination tests were confirmed by the tube agglutination test with monospecific sera. Suspensions of the standard strains of *B. abortus* and *B. melitensis* were always included as controls.

RESULTS

The pattern of results obtained with the three F.A.O./W.H.O. reference strains of *Brucella*, together with *Brucella abortus* strain 19 (the vaccine strain), is shown in Table 1. *Brucella abortus* strain 19 did not grow at concentrations of 1/500,000 and 1/1,000,000 of thionin blue; the reference strains *B. abortus*, *B. melitensis* and *B. suis* did grow at these concentrations. At the 1/2,000,000 concentration of thionin blue, *B. abortus* strain 19 did grow, but in all cases gave only 2+ to 3+ values. *Brucella melitensis* and *B. suis* also showed less growth at the 1/500,000 concentration of thionin blue. A number of recently isolated *Brucella* cultures was examined, using the three concentrations of thionin blue; a concentration of 1/500,000 was eventually chosen for routine use, since this gave clear-cut results.

In Table 2 are given the results of the examination of 171 cultures of *Brucella*, together with their source, and it is apparent that there were three groups of *Brucella abortus* which did not grow on thionin blue at concentrations which allowed the growth of the majority of the strains. Of these, the largest group (15·2% of

Table 1. *The results of examining standard strains of Brucella*

Strain	CO ₂ require- ment	Growth on serum agar plus					Agglutination in monospecific abortus/ melitensis sera
		Basic fuchsin 1/25,000	Thionin 1/50,000	Thionin blue			
				1/ 500,000	1/ 1,000,000	1/ 2,000,000	
<i>B. abortus</i> strain 544 (reference strain)	+	5+	—	5+	5+	5+	+
<i>B. abortus</i> strain 19 (vaccine strain)	—	5+	—	—	—	2+	+
<i>B. melitensis</i> 16M (reference strain)	—	5+	5+	3+	5+	5+	+
<i>B. suis</i> 1330 (reference strain)	—	—	5+	2+	5+	5+	+

Table 2. *The results of examining 171 Brucella isolates*

Organism	CO ₂ requirement	Growth on serum glucose agar plus				Slide agglutination with monospecific abortus/melitensis sera	No. of strains tested	Source distribution of strains
		Basic fuchsin (1/25,000)	Thionin blue (1/500,000)		Thionin (1/50,000)			
Typical <i>B. abortus</i>	+	5+	—	5+	+	—	118	51 foetal, 67 milk
Aerobic <i>B. abortus</i>	—	5+	—	5+	+	—	2	2 milk
<i>B. abortus</i> type II (Wilson)	+	—	—	—	+	—	26	19 milk, 7 foetal
<i>B. abortus</i> Vaccine strain 19	—	5+	—	—	+	—	8	3 foetal, 5 milk
<i>B. abortus</i> Thionine blue sensitive	+	5+	—	—	+	—	7	5 foetal, 2 milk
<i>B. abortus/melitensis</i>	+	5+	—	5+	—	+	2	2 foetal
<i>B. abortus</i> thionin-resistant	—	5+	5+	5+	+	—	1	1 foetal (Holland)
<i>B. melitensis</i>	—	5+	5+	3+	—	+	7	2 foetal, 5 milk

all cultures studied) belonged to the type II (Wilson). Such strains are known to be sensitive to most dyes. A second group, comprising 4.7 % of all strains examined, behaved biochemically exactly like the vaccine strain 19 *B. abortus*; these were recovered both from foetal material and from milk.

The third group (4.1 % of total isolates examined) consisted of strains that did not grow in presence of thionin blue but differed from *Brucella abortus* strain 19 in requiring additional CO₂ for growth; but they did grow in presence of basic fuchsin, thus differing from *B. abortus* type II (Wilson). Such strains were also recovered from milk and foetal material. Most of the strains (69 %) were typical of *B. abortus* in all their reactions, but two aerobic strains were also recovered. The other cultures consisted of *B. melitensis* (4.1 %) and *B. abortus/melitensis* (2.1 %). One strain of thionin-resistant *B. abortus* was received (from Holland).

Cultures obtained from guinea-pigs and the milk of cows experimentally infected with *Brucella abortus* strain 19, and with the thionin blue sensitive strains of *B. abortus* in all cases behaved exactly like the strains used for inoculation, thus confirming their stability *in vivo*.

At the 1/500,000 concentration of thionin blue, a few colonies were observed with strain 19 and with some of the CO₂ requiring thionin blue sensitive strains. After subculture on serum agar these resistant colonies gave profuse growth (5+ readings), indistinguishable from those of the standard *Brucella abortus* 544 on thionin blue at a dilution of 1/500,000.

DISCUSSION

Widespread vaccination of cattle with *Brucella abortus* strain 19 is carried out in this and other countries, and many workers have drawn attention to the need for a simple and reliable method for the differentiation of the vaccine strain from other strains of *B. abortus*. However, no single character can be singled out as characteristic of the vaccine strain only, and it is obvious that the thionin blue sensitivity test is not pathognomonic for strain 19. In addition to *B. abortus* strain 19 and *B. abortus* type II (Wilson), a third group has now been found, also sensitive to thionin blue. Cultures of this group differ from strain 19 in that they need added CO₂ for growth. Preliminary experiments at this laboratory on the virulence of cultures of the thionin blue sensitive group of *B. abortus* have shown that these are as virulent as the standard 544 strain. Virulence tests were based on the duration of bacteraemia in guinea-pigs (Cruickshank, 1957).

Since *Brucella abortus* strain 19 does not appear in the milk of cows after subcutaneous inoculation (Taylor & McDiarmid, 1949) the origin of strains recovered both from milk and foetuses, and indistinguishable from strain 19, is a matter for conjecture. Some of these strains, together with strain 19, have been examined for virulence in guinea-pigs, and all have been of equally low virulence. The only conclusion appears to be that either strain 19 can appear in the milk and foetal material in some cases, or that strains occur in the field that are indistinguishable from strain 19, by our present methods.

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5-Aminoimidazole and its Riboside from Biotin-Deficient Yeast

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SUMMARY

A purified preparation of the 'amine' which accumulated in the medium during the growth of a strain of *Saccharomyces cerevisiae* (yeast 47) under conditions of partial biotin-deficiency contained two compounds, one of which was chromatographically and electrophoretically identical with synthetic 5-aminoimidazole. Both compounds gave identical colours on diazotization and coupling in the Bratton & Marshall reaction and on treatment with the Pauly imidazole reagent. The other, and major, component of purified 'amine' was shown to be 5-aminoimidazole riboside by its conversion to the free base and ribose on incubation with a bacterial nucleosidase. Synthetic 5-aminoimidazole gave analytical figures for formate, $\text{NH}_3\text{-N}$ and total-N similar to those previously obtained for purified 'amine'. Evidence was obtained that resting organisms of a mutant *Escherichia coli* converted 'amine' and synthetic 5-aminoimidazole to 5-amino-4-imidazole-carboxamide. One of the adenine derivatives which accumulated instead of 'amine' and hypoxanthine in a medium supplemented with aspartate was tentatively identified as thiomethyladenosine.

INTRODUCTION

Previous papers (Chamberlain, Cutts & Rainbow, 1952; Chamberlain & Rainbow, 1954; Lones, Rainbow & Woodward, 1958) described the accumulation of diazotizable amine ('amine') and hypoxanthine in culture filtrates of *Saccharomyces cerevisiae* (yeast 47) after growth in a biotin-deficient defined medium which contained L-methionine. 'Amine' accumulation was invariably associated with the formation of a pink pigment by the organism. Addition of adenine or aspartate to the medium suppressed the production of this pigment and the accumulation of 'amine' and hypoxanthine. These results were regarded as indicating that a derangement in adenine biosynthesis was conditioned by biotin deficiency. Preliminary studies on a purified preparation indicated that 'amine' was 5-aminoimidazole riboside. In the present paper, evidence is presented which, in conjunction with that already reported, identifies beyond reasonable doubt the chief components of purified 'amine' as 5-aminoimidazole and its riboside, the latter being the major component.

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METHODS

Preparation of 'amine'. Purified 'amine' was prepared from culture filtrates of *Saccharomyces cerevisiae* (yeast 47) grown on a defined medium containing DL-methionine (500 $\mu\text{g./ml.}$) and D-biotin (0.02 $\text{m}\mu\text{g./ml.}$). Details of the organism, its maintenance and cultivation, and of the purification of 'amine' were as given by Lones *et al.* (1958), except: (a) the final eluate was concentrated by lyophilization; (b) by using proportionately larger ion-exchange resin columns, a total of 2 l. culture filtrate, yielding 28–38 mg. crystalline material, was handled. These preparations are referred to as purified 'amine' and were used as the chief experimental material in this work.

Preparation of synthetic 5-aminoimidazole. An aqueous solution of 5(4)-aminoimidazole was prepared freshly as required by catalytic reduction of 5(4)-nitroimidazole (Rabinowitz, 1956).

Experiments with suspension of organisms. Resting suspensions of *Escherichia coli* B 96 were used, according to the procedure described by Gots (1950), to demonstrate the conversion *in vivo* of 'amine' to 5(4)-amino-4(5)-imidazolecarboxamide, which accumulates with this purineless mutant. The organism was grown for 24 hr. at 37° in 200 ml. portions of a medium consisting of glucose (0.2%, w/v), tryptone (2%, w/v), and hypoxanthine (20 $\mu\text{g./ml.}$). The organisms were harvested by centrifugation, washed twice with 0.85% (w/v) saline and resuspended in 1 ml. 0.85% (w/v) saline.

Complete reaction mixtures (6.1 ml.) contained 0.13 M-phosphate buffer (pH 7.2; 3 ml.), 0.02 M-glucose (3 ml.), purified 'amine' solution (20 $\mu\text{l.}$) and organism suspension (0.1 ml.). Reaction mixtures were incubated for 3 hr. at 37°, after which the organisms were centrifuged down, and the clear supernatant fluids submitted to the Bratton & Marshall diazotization procedure (see below). The absorption maxima of the colours thus produced were determined spectrophotometrically.

Preparation of bacterial nucleosidase. The bacterial nucleosidase used in this work was an unpurified extract of *Lactobacillus brevis* L4. This organism was grown on 500 ml. portions of the medium described by Chamberlain & Rainbow (1954) except that glucose was omitted and maltose monohydrate (2%, w/v) and $\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$ (0.4%, w/v) were included. After incubation at 28° for 24 hr., the organisms were harvested (centrifuge), washed twice with 1% (w/v) KCl solution, resuspended in 5 ml. of that solution, and then disrupted in a tissue disintegrator (H. Mickle, Gomshall, Surrey) with ballotini beads (0.2–0.3 mm. diameter). During the process the temperature was kept below 5° by alternating cycles (5 min.) of shaking and cooling in ice. The disrupted suspension was poured off from the beads and centrifuged at 2° to remove cell debris. The supernatant fluid was used immediately, or stored at –20° at which temperature it retained its nucleosidase activity for at least 2 months. This extract readily hydrolysed adenosine to adenine and ribose.

Analytical methods

Diazotizable amine. This was determined by the colour produced in the Bratton & Marshall diazotization and coupling procedure as described by Chamberlain & Rainbow (1954).

Pauly reaction. This was performed on solutions in accordance with the modification suggested by Koessler & Hanke (1919) or on paper by the modification introduced by Ames & Mitchell (1952).

Ribose. In solutions this was determined by the orcinol method of Drury (1948). On paper chromatograms and electrophoretograms, free ribose was detected by aniline hydrogen phthalate and compared with authentic D-ribose as marker. Combined ribose was detected directly on paper by the lead tetra-acetate method of Buchanan, Dekker & Long (1950) and, after elution, by the orcinol method.

Purine and purine derivatives. These were detected on paper chromatograms and paper electrophoretograms by ultraviolet photography. After elution, absorption spectra were determined in an S.P. 500 quartz spectrophotometer (Unicam, Cambridge).

Paper chromatography. A conventional descending solvent technique, with Whatman no. 3 or no. 4 paper and (unless otherwise stated) the butanol + acetic acid + water (125 + 30 + 125) solvent of Woiwod (1949), was used.

Paper electrophoresis. This was carried out in 0.05 M-buffer solutions, with 22 in. strips of Whatman no. 3 paper in an enclosed strip technique. Voltages of 2000–3000 V. were usually used.

RESULTS

Chromatographic and electrophoretic behaviour of purified 'amine'

When subjected to paper chromatography or paper electrophoresis samples (about 500 μ g.) of purified 'amine' gave two major diazotizable spots (see also Lones *et al.* 1958). Table 1 summarizes some of the properties of the components thus separated.

Table 1. *Properties of two diazotizable materials separable from purified 'amine'*

R_F values are given for descending chromatograms on Whatman no. 4 paper with solvent *n*-butanol + acetic acid + water (125 + 30 + 125). Migrations are quoted as cm. moved in 0.05 M-acetate buffer (pH 5.0) when 3000 V. was applied for 50 min. Pentose was tested for by the lead tetra-acetate and the orcinol reactions; phosphate by Allen's (1940) method.

Com- ponent	R_F value	Electro- phoretic migration	Bratton & Marshall colour (λ_{max} ; $m\mu$)	Pauly colour	Pentose	Phosphate
Major	0.33	22.5	Orange-red (500)	Yellow	Present	Absent
Minor	0.46	37.7	Orange-red (500)	Blue	Absent	Absent

The results indicated that the slower-moving compound (chromatographically; electrophoretically) was an imidazole riboside derivative, while the faster-moving one was the corresponding free base, as Lones *et al.* (1958) had found. The riboside was quantitatively the major constituent. Supporting evidence was obtained by electrophoresis at different pH values. At more acid pH values, the ionization of the diazotizable amino group was enhanced and migration increased: at less acid pH values, the opposite behaviour was observed (Table 2). The results with borate buffer (pH 10) were particularly significant. This buffer has been used for the electrophoretic separation of sugars (Foster, 1952), the borate ion complexing with

the free hydroxyl groups of the sugar and giving good migrations towards the anode. In borate buffer the migration of the riboside was reversed, while the free base continued to migrate towards the cathode but at a much decreased rate because of the suppression of ionization of the amino group at pH 10. Ascending chromatography in a solvent consisting of 85 % (w/v) ammonium bicarbonate in water (Hems, 1959) also reversed the order in which the diazotizable spots separated. This behaviour was consistent with the proposed relationship between the two substances, the presence of the ribose moiety conferring on the riboside more hydrophilic properties.

Table 2. *Electrophoretic behaviour of the diazotizable constituents of purified 'amine' preparations*

Spots (20 μ l.) were applied to Whatman no. 4 paper and migration measured after 50 min. application of 3000 V.

Buffer	Migration			
	Riboside		Free base	
	(cm.)	direction	(cm.)	direction
Formate (pH 3.5)	31.5;	to cathode	51.5;	to cathode
Acetate (pH 5.0)	22.5;	to cathode	37.7;	to cathode
Phosphate (pH 7.0)	10.3;	to cathode	15.7;	to cathode
Borate (pH 10.0)	11.3;	to anode	6.4;	to cathode

Identity of the free-base component of 'amine' preparations with 5(4)-aminoimidazole

Synthetic 5-aminoimidazole gave a strong orange-red Bratton & Marshall colour with an absorption maximum (500 $m\mu$) identical with that given by purified 'amine' preparations. The chromatographic mobilities of the synthetic material and the free-base constituent of 'amine' were identical, as were their electrophoretic migrations at pH values 3.5, 5.0, 7.0 and at pH 10.0 (in borate buffer). In the Pauly reaction, the synthetic material and the free-base component gave identical blue colours.

The ultraviolet absorption spectrum of the purified 'amine' used in this work differed slightly from that reported by Lones *et al.* (1958) in that the feeble peak at 238 $m\mu$ was absent. The elimination of this peak may have been the result of the somewhat improved method of 'amine' preparation used.

Samples of synthetic 5-aminoimidazole were hydrolysed and then analysed for formic acid, ammonia and total-N by the methods described by Lones *et al.* (1958). For two different preparations the molar ratios of formate: $\text{NH}_3\text{-N}$: total-N (referred to $\text{NH}_3\text{-N}$ as 2) were 1:2.4 and 0.93:2:4. Considering the great instability of 5-aminoimidazole and that only unpurified aqueous solutions were available for analysis, these values compared fairly well with the theoretical ratio of 1:2:3, and with the ratios obtained by Lones *et al.* (1958) for purified 'amine'. The possible presence of small quantities of unchanged nitroimidazole may be a cause of the high figures for total-N in the synthetic material.

Enzymic hydrolysis of the riboside component

Portions of purified 'amine' solution containing about 400 $\mu\text{g.}$ were incubated for 24 hr. with 0.5 ml. *Lactobacillus brevis* L4 extract. After concentration *in vacuo* at room temperature, samples of the reaction mixtures, together with reference spots of D-ribose and synthetic 5-aminoimidazole, were chromatographed and examined for diazotizable compounds and for free ribose. The chromatograms showed that extensive but not complete hydrolysis of 'amine' had occurred, the amount of free-base component and of free ribose had increased, and the amount of riboside component had diminished. These changes did not take place when boiled L4 extract was substituted for unboiled extract. Under the above conditions adenosine, guanosine, inosine, xanthosine and cytidine underwent extensive hydrolysis in 3 hr. by the bacterial extract, whereas a much longer period (12 hr.) was required before the riboside component of 'amine' underwent appreciable hydrolysis. This slow rate of action may reflect the pseudo-nucleoside structure of the 'amine' riboside substrate, in which an imidazole derivative takes the place of the 'enzymically preferable' purine or pyrimidine moiety of normal nucleosides.

Table 3. *Conversion of 'amine' to a new diazotizable substance by Escherichia coli B96*

The complete reaction mixture contained: 0.13 M-phosphate buffer (pH 7.2; 3.0 ml.); 0.02 M-glucose (3.0 ml.); purified 'amine' solution (about 600 $\mu\text{g.}$ in 20 $\mu\text{l.}$); 0.1 ml. washed suspension of *E. coli* B96. The colour was that obtained when the Bratton & Marshall procedure was applied to the supernatant fluids after reaction had proceeded for 3 hr. at 37°.

Reaction mixture	Colour	
	Visual	(λ_{max} , $\text{m}\mu$)
Complete	Purple	520
'Amine' omitted	Colourless	—
Organisms omitted	Orange-red	499
Phosphate omitted	Orange-red	499
Glucose omitted	Orange-red	500
Complete + boiled organisms	Orange-red	499

*Conversion of 'amine' to 5-amino-4-imidazole
carboxamide in vivo*

The change in colour developed on diazotization and coupling with the Bratton & Marshall reagents has been used as an index of the conversion of 5-aminoimidazole to 5-amino-4-imidazolecarboxamide (Love & Gots, 1955; Friedman & Moat, 1958). The changes which occurred in the reaction mixture when freshly prepared 'amine' solutions were incubated with washed suspensions of *Escherichia coli* B96 are recorded in Table 3 which shows that, in a complete system for which living organisms, glucose and inorganic phosphate were all essential, there was produced diazotizable material giving a purple colour, quite distinct from the orange-red colour given by 'amine' itself. The absorption maximum of this material (520 $\text{m}\mu$) which gave the purple colour was nearer that of 5-amino-4-imidazolecarboxamide (525 $\text{m}\mu$) than of 'amine' (500 $\text{m}\mu$) so that the change observed was consistent with

a substantial conversion of 'amine' to 5-amino-4-imidazolecarboxamide. Similar changes occurred when synthetic 5-aminoimidazole was substituted for 'amine' in the complete reaction system. No purple colour resulted when 'amine' or synthetic 5-aminoimidazole was omitted from the reaction mixtures, i.e. the changes did not represent new synthesis of 5-amino-4-imidazolecarboxamide.

The complete conversion of 'amine' or synthetic 5-aminoimidazole to 5-amino-4-imidazolecarboxamide, as indicated by azo colour with an absorption maximum of $525\text{ m}\mu$, was not obtained even when complete reaction mixtures were incubated for longer periods. However, artificial mixtures of 'amine' and synthetic 5-aminoimidazole gave colours having a single absorption maximum intermediate between the maxima given by either component singly, the actual value depending on the relative proportions of the components. These results are consistent with the belief that 'amine' is 5-aminoimidazole (or a near derivative) and that it undergoes transformation to 5-amino-4-imidazolecarboxamide by living *Escherichia coli* B96.

'Amine' and pigment formation

Purified 'amine' was a pale greenish yellow crystalline solid which slowly darkened with the formation of deep purplish pigments which settled out of solution in small flocculent masses even during storage at $0-4^\circ$ *in vacuo* in the dark. This darkening and precipitation of pigments was most marked in relatively concentrated aqueous solutions ($5-20\text{ }\mu\text{g./ml.}$) and was always associated with loss of diazotizability. On chromatographic or electrophoretic development on paper, 'amine' left a trail of apparently similar purplish pigments; solutions of synthetic 5-aminoimidazole behaved similarly. The properties and manner of formation of these pigments indicate them to be substances of high molecular weight formed by polymerization involving the primary (diazotizable) amino group of 5-aminoimidazole. Hunter & Hlynka (1941) produced evidence that pigment production from 5-aminoimidazole involved deamination followed by oxidative condensations; during the course of the present work it was observed that a remarkable series of yellow-orange and red zones appeared during the chromatographic development of relatively large amounts of synthetic 5-aminoimidazole. All these substances were less mobile than 5-aminoimidazole itself and may well have represented different stages of polymer formation. The readiness with which 'amine' undergoes transformation into pigments suggests that the pink pigment characteristic of *Saccharomyces cerevisiae* yeast 47 when it is accumulating 'amine' is derived from 'amine' itself.

Formation of thiomethyladenosine by Saccharomyces cerevisiae, yeast 47

Lones *et al.* (1958) showed that the addition of L-aspartate prevented the accumulation of 'amine' and hypoxanthine during the growth of *Saccharomyces cerevisiae* yeast 47 on their medium II; instead, adenine derivatives accumulated. One of these compounds was tentatively identified as thiomethyladenosine as follows. Concentrations of the adenine-containing compounds prepared according to Lones *et al.* (1958) were submitted to separation by paper chromatography; the fastest-moving ultraviolet-absorbing spot (R_F value about 0.8) was located, cut out and eluted. Various eluates were prepared and the material in them was shown to have the

following properties: (a) it gave positive reactions in the modified nitroprusside test of Csonka & Denton (1946), and in Feigl's (1939) test for the $=C=S$ or $\equiv C-SH$ linkage; (b) on hydrolysis with $2N-H_2SO_4$ at 100° for 2 hr. it yielded a single purine derivative chromatographically and spectrophotometrically identical with adenine; (c) its ultraviolet spectrum in $0.1N-HCl$ was indistinguishable from that of authentic adenosine (λ_{max} , $257 m\mu$); (d) on electrophoresis in $0.05M$ -phosphate buffer (pH 7.0) it migrated slowly towards the cathode ($0.066 cm.hr.^{-1}/V.cm.^{-1}$ as compared with $0.070 cm.hr.^{-1}/V.cm.^{-1}$ quoted for thiomethyladenosine by Baddiley, Cantoni & Jamieson, 1953); (e) it gave a positive but weak response in the orcinol pentose test; this accords with the report of Baddiley *et al.* (1953) that only 32% of the pentose present in thiomethyladenosine was detected after 40 min. heating in this test; (f) it contained no phosphorus detectable by Allen's (1940) method after preliminary incineration with 60% (w/v) perchloric acid. The absence of phosphate was also confirmed by its electrophoretic behaviour.

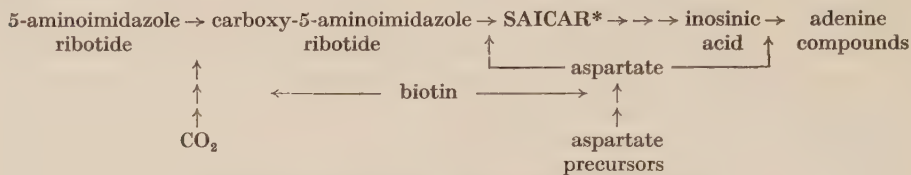
DISCUSSION

Taken in conjunction with previous work (Chamberlain & Rainbow, 1954; Lones *et al.* 1958) the present work establishes the identity of the diazotizable amine accumulated by *Saccharomyces cerevisiae* yeast 47 under conditions of biotin-deficiency as a mixture of 5-aminoimidazole riboside and a smaller quantity of the aglycone. It was not possible to determine whether the free base was present as such in the culture filtrate or whether it arose by hydrolysis during purification since the Bratton & Marshall test did not distinguish between riboside and free base. The presence of traces of other diazotizable substances is not excluded.

The role of 5-aminoimidazole as an intermediate in purine biosynthesis at the nucleotide level is now well substantiated (Love & Gots, 1955; Levenberg & Buchanan, 1956; Lukens & Buchanan, 1959). In the light of this knowledge, the accumulation of 'amine' hypoxanthine by yeast 47 may be interpreted as due to a block in aspartate synthesis conditioned by biotin-deficiency. First, the conversion of inosinic acid to adenylic acid involves the amino group of aspartate (Abrams & Bentley, 1955). Secondly, the conversion of 5-aminoimidazole ribotide to 5-amino-4-imidazole(*N*-succinyl)-carboxamide ribotide via the intermediate 5-amino-4-imidazolecarboxylic acid ribotide involves both aspartate and the incorporation of CO_2 (Lukens & Buchanan, 1959). Biotin is known to be involved in aspartate metabolism in yeast (Winzler, Burk & du Vigneaud, 1944) and in bacteria (Lichstein & Umbreit, 1947; Broquist & Snell, 1951) and in bacterial CO_2 fixation (Broquist & Snell, 1951). Hence, biotin deficiency may well cause hypoxanthine, 5-aminoimidazole and its riboside to be excreted as the respective degradation products of the inosinic acid and 5-aminoimidazole ribotide (or the readily decarboxylated carboxy-5-aminoimidazole ribotide) which accumulate initially within the cell.

A supply of exogenous aspartate removes these biotin-dependent blocks in purine biosynthesis, and then adenine-containing compounds accumulate instead of 'amine' and hypoxanthine. The presence of thiomethyladenosine as one of these compounds is readily explained since it is a degradation product of S-adenosyl-methionine, which accumulates in yeast cells grown on methionine-rich media

(Schlenk & DePalma, 1957). The following scheme illustrates the proposed relationships:



* 5-amino-4-imidazole-(*N*-succinyl)-carboxamide.

Moat *et al.* (1956) have also reported the accumulation of diazotizable amine, believed to be 5-aminoimidazole riboside (Friedman & Moat, 1958), by a biotin-dependent strain of *Saccharomyces cerevisiae* when growing in the presence of methionine; as with *S. cerevisiae* yeast 47, adenine and aspartic acid inhibited its accumulation but, unlike yeast 47, 'amine' accumulation was accompanied by that of inosine and not hypoxanthine.

The role of methionine in 'amine' formation is not clear. Methionine greatly enhances 'amine' production but it has no obvious effect on hypoxanthine accumulation, which appears to be solely the result of the imposed biotin deficiency. In liver slices methionine can give rise to formate and serve as a source of the β -carbon of serine (Siekevitz & Greenberg, 1950). In 'amine' synthesis with *Saccharomyces cerevisiae* yeast 47, methionine possibly plays a similar role in generating the one-carbon fragment required for the conversion of glycinamide ribotide to formylglycinamide ribotide, a precursor of 5-aminoimidazole ribotide.

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Germination Under Alkaline Conditions and Transmission of Alkali Resistance by Endospores of Certain Strains of *Bacillus cereus* and *Bacillus circulans*

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SUMMARY

Endospores of *Bacillus cereus* Frankland and Frankland strain R (which is able to grow under highly alkaline conditions) germinated at a higher pH value than spores of an alkali-sensitive strain, Mu-3055. Spores of *B. circulans* Jordan strain Ru 38 (able to grow at pH 11.0) germinated at pH 11.0. Spore germination at any pH value was generally followed by outgrowth to the vegetative rod. Outgrowth of germinated spores of *B. cereus* R at pH 10.0 and of those of *B. circulans* Ru 38 at pH 11.0 took place at 30° but not at 37°. Growth from a vegetative inoculum of *B. cereus* R at pH 10.0 or 10.3 took place more readily at 30° than at 37°. The ability of the vegetative forms of both bacterial species to grow under alkaline conditions was transmitted through the endospore.

INTRODUCTION

Two strains of alkali-resistant *Bacillus* species have recently been described: a strain of *B. cereus* Frankland and Frankland able to grow in media of pH 10.3 (Kushner & Lisson, 1959), and a strain of *B. circulans* Jordan able to grow at pH 11.0 (Chislett & Kushner, 1961). Previous work was largely concerned with the effect of alkaline media on the vegetative growth of these organisms, but gave no information about the effect of such alkaline media on endospore germination. We have now examined the effect of alkaline conditions on the development of the vegetative form from the endospore and have examined how the ability of the vegetative form to grow under alkaline conditions is transmitted through the endospore.

METHODS

Organisms. *Bacillus cereus* strain Mu-3055 (non-adapted: sensitive to alkali) and *B. cereus* strain R (adapted from Mu-3055 and resistant to alkali); both described by Kushner & Lisson (1959); *B. circulans* strain Ru 38 (maintained routinely on buffered nutrient agar (pH 7.4) but capable of growth at pH 11.0); described by Chislett & Kushner (1961).

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Culture media. The media used were: phosphate-buffered nutrient broth (BNB) and phosphate-buffered nutrient agar (BNA) of final pH values 7.4 and higher, prepared as before (Kushner & Lisson, 1959; Chislett & Kushner, 1961).

Preparation of spore suspensions. The three strains were subcultured several times in BNB (pH 7.4), with incubation at 30°, before finally spreading 1 ml. of 6 hr. culture on the surface of 50 ml. BNA (pH 7.4) in a 20 oz. bottle; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ had been added to the BNA medium to a final concentration of 5 p.p.m. Mn^{++} , and additional agar (Davis, New Zealand) was added to give a final concentration of 3% (w/v) agar in the medium. The *Bacillus cereus* strains were incubated at 30° for 7 days and the *B. circulans* strain for 14 days. Spores were harvested by washing off the growth with sterile distilled water and glass beads. The resulting suspension was washed six times in sterile distilled water at 5° and the deposit, consisting almost entirely of spores, resuspended in 0.01 M-phosphate buffer (pH 7.4). After making viable spore counts on nutrient agar plates, the spore suspensions were diluted with 0.01 M-phosphate buffer (pH 7.4) to contain 10^9 viable spores/ml. and held at 5° until required. Before making viable counts and before use in the experiments described in Table 1, the portion of each spore suspension used was heated at 80° for 15 min., then cooled rapidly in an ice bath and held at 5° until required. In the experiments shown in Table 2 and in those with shaken cultures of *B. circulans*, the spores used had been prepared in a similar manner, with the following variations in technique: BNA medium (pH 7.4) with 5 p.p.m. Mn^{++} added was used; incubation was for 7 days at 37° instead of at 30°. At the end of this time there was a high degree of sporulation and comparatively few vegetative forms remained. The washed suspensions of spores + bacteria were heated for 1 hr. at 60°, adjusted to a concentration of 3×10^8 viable spores/ml. and stored in distilled water at 5°.

Germination of spores at pH 7.4 and at alkaline pH values. Spore suspensions were added to 10 ml. BNB medium in 6 in. \times $\frac{5}{8}$ in. tubes at pH values from 7.4 to 11.0, to give a final concentration of 10^7 viable spores/ml. Incubation was at 30° and 37°, and examinations were made at 2, 4, 6, 8, 10, 12 and 24 hr. Spore germination was detected by examining the suspension in a haemocytometer, by using a 4 mm. phase contrast objective (see Berger & Marr, 1960). Under phase contrast, the ungerminated spores appeared refringent, thus allowing a direct spore count. The staining method of Conklin (1934) with malachite green and mercurochrome, whereby ungerminated spores stained green and germinated spores and vegetative forms red, gave a good correlation with the results obtained by the haemocytometer and phase contrast method. For quantitative determinations of the number of germinated and ungerminated spores/ml., and to estimate percentage germination, 0.01 ml. spore suspension was delivered by a dropping pipette on to a marked area of 1 cm.² on a slide and the suspension stained by Conklin's method. As soon as microscopic observation showed that the spores were germinating, the pH values of the cultures and of control tubes of uninoculated media were measured. The results have been recorded as the relative extent of germination at the earliest time this process was observed (see Table 1). Once germination of even a small proportion of spores had taken place, subsequent vegetative cell growth lowered the pH value of the medium (Kushner & Lisson, 1959; Chislett & Kushner, 1961). For this reason, information about the time-course of germination of a spore population at a given pH value could not readily be obtained in these experiments.

Outgrowth. The successive changes leading to the formation of a vegetative rod from a germinated spore have been termed 'outgrowth' (Campbell, 1957). This process has been recorded here qualitatively simply by noting the first appearance of vegetative rods in the culture medium.

Transmission of alkali resistance. Growing cultures in BNB medium (pH 7.4) resulting from spore germination at this pH value were inoculated into duplicate tubes of BNB medium at different pH values between 7.4 and 11.0. The inocula were two 4 mm. loopfuls of an 18–21 hr. culture at 37°. The tubes were incubated at 30° or 37° and examined at intervals. As soon as growth was visible as turbidity the cultures were examined microscopically, and pH measurements were made of the cultures and of control tubes of uninoculated media.

RESULTS

Spore germination and outgrowth

At pH 7.4, both alkali-sensitive and alkali-resistant strains of *Bacillus cereus* showed considerable germination and outgrowth after 2 hr. at 30° or 37° (Table 1). At pH 9.5, spore germination and outgrowth took place much more rapidly and extensively with the alkali-resistant strain than in the alkali-sensitive strain. Both processes were seen at pH 10.0 with *B. cereus* R, but not with *B. cereus* Mu-3055. Germination did not take place at pH values higher than 10.0 with either strain. At the highest pH values at which germination was possible for each strain, this process and/or outgrowth took place more slowly, if at all, at 37° than at 30°.

Table 1. *Germination and outgrowth of spores of alkali-sensitive and alkali-resistant Bacillus cereus and of alkali-resistant B. circulans*

Organism	pH value	Temperature	Spore germination*	Outgrowth†
<i>B. cereus</i> Mu 3055 (alkali-sensitive)	7.4	30°	+++ at 2 hr.	+ at 2 hr.
		37°	+++ at 2 hr.	+ at 2 hr.
	9.5	30°	(+) at 4 hr.	+ at 4 hr.
		37°	(+) at 24 hr.	+ at 24 hr.
	10.0, 10.3	30°	— at 24 hr.	— at 24 hr.
		37°	— at 24 hr.	— at 24 hr.
<i>B. cereus</i> R (alkali-resistant)	7.4	30°	+++ at 2 hr.	+ at 2 hr.
		37°	+++ at 2 hr.	+ at 2 hr.
	9.5	30°	+++ at 2 hr.	+ at 4 hr.
		37°	++ at 2 hr.	+ at 4 hr.
	10.0	30°	(+) at 12 hr.	+ at 24 hr.
		37°	(+) at 12 hr.	— at 24 hr.
	10.3, 10.7	30°	— at 24 hr.	— at 24 hr.
		37°	— at 24 hr.	— at 24 hr.
<i>B. circulans</i> Ru 38 (alkali-resistant)	7.4	30°	+ at 2 hr.	+ at 4 hr.
		37°	+ at 2 hr.	+ at 4 hr.
	9.5, 10.0,	30°	+++ at 2 hr.	+ at 2 hr.
		37°	+++ at 2 hr.	+ at 2 hr.
	10.3	30°	+ at 2 hr.	+ at 4 hr.
		37°	+ at 2 hr.	+ at 4 hr.
	10.7	30°	+ at 2 hr.	+ at 4 hr.
		37°	+ at 4 hr.	+ at 4 hr.
	11.0	30°	+ at 4 hr.	— at 24 hr.

* % spores germinated reported as follows: (+) 5 %, + 5–20 %, ++ 20–50 %, +++ > 50 %.

† Outgrowth = appearance of rods at time indicated.

The results obtained with spores of the alkali-resistant *Bacillus circulans* strain Ru 38 differed markedly from those obtained with the *B. cereus* strains in that germination was more extensive after 2 hr. at pH values from 9.5 to 10.3 than at pH 7.4. Outgrowth was observed within 2 hr. with the pH range 9.5–10.3, but was not observed until 4 hr. at pH 7.4. Results at pH 10.7 or 11.0 were similar to those found at pH 7.4, except that at pH 11.0, outgrowth took place at 30° but not at 37°.

Transmission of alkali resistance

Following spore germination at pH 7.4, the vegetative form of *Bacillus cereus* strain Mu-3055 in standing tubes showed growth after 4 hr. at pH 7.4 and pH 9.5, at 30° and at 37°; no growth occurred at pH 10.0 or higher. *B. cereus* strain R showed growth after 4 hr. at pH 7.4 and pH 9.5, at 30° and at 37°. At pH 10.0 and pH 10.3, growth was observed within 12 hr. at 30°, but not until 24 hr. at 37°. *B. circulans* strain Ru 38 showed growth after 4 hr. at 37° in the pH range 7.4 to 11.0, but at 30° growth was not observed in this pH range until after 6 hr. incubation.

Table 2. *Effect of pH value on the growth of alkali-sensitive and alkali-resistant Bacillus cereus following spore germination at pH 7.4*

In Expts. 2 and 3 one drop of spore suspension of strain Mu-3055 or strain R was added to 10 ml. BNB (pH 7.4) medium and incubated 4 hr. with shaking at 37°, to an optical density of 0.7–0.8. Three drops of each culture were added to tubes of BNB medium of different pH values. Shaking and measurements of optical density were as described by Kushner & Lisson (1959).

Data for Expt. 1 from Table 3 of Kushner & Lisson (1959).

Expt.	Inoculum	pH value	Sensitive (strain Mu-3055)	Resistant (strain R)
			$T_{0.4}$ (hr.)*	
1	Bacteria maintained on plates (mixture of spores and vegetative forms)	9.5	16	7.2
		9.8	> 25	11.0
		10.0	> 25	24.8
		10.1	> 25	> 25
2	Bacteria obtained by spore germination, 2 weeks after preparation of spores	9.4	9	5
		9.8	> 24	10
		9.9	> 24	20
3	Bacteria obtained by spore germination, 16 weeks after preparation of spores	9.4	10	4
		9.8	> 24	21
		9.9	> 24	> 24

* Time at which cultures reached a (net) optical density of 0.4.

An examination of the effects of pH value on the vegetative growth of *Bacillus cereus* derived from spores was also made in shaken cultures at 37° (Table 2). Once growth had begun there was, as before noted (Kushner & Lisson, 1959), little effect of the initial pH value on the growth rate; and the growth rates observed were similar to those reported by Kushner & Lisson (1959). Thus vegetative forms derived from spores 2 weeks after sporulation had retained full alkali resistance. After an additional 14 weeks storage of the spores in distilled water at 5°, however, there was some loss of alkali resistance; the vegetative forms from germinated spores were no longer able to grow at pH 9.9 in 24 hr.

In an experiment with spores of the alkali-resistant *Bacillus circulans*, an inoculum of the vegetative form was prepared by incubating these spores overnight on a BNA medium plate (pH 7.4), and a series of tubes containing BNB medium of different pH values was inoculated and shaken as before (Chislett & Kushner, 1961). The growth curves obtained were virtually identical with those given by the alkali-resistant *B. circulans* which had been maintained at pH 7.4 and transferred as a mixture of spores and vegetative forms (Chislett & Kushner, 1961, Fig. 1*a*).

These results indicated that growth of the alkali-resistant bacteria took place at a slightly higher pH value in standing than in shaken cultures. However, in standing cultures at 30° and 37° *Bacillus cereus* R showed growth at pH 10.3 (as above) but not at pH 10.7; and *B. circulans* Ru 38 showed growth at pH 11.0 (as above) but not at pH 12.0. The highest pH value at which growth was observed for *B. circulans* Ru 38 was pH 11.4 (pH value of standing culture) after 18 hr. at 37°.

DISCUSSION

In obtaining *Bacillus cereus* strain R the culture method used was designed to select vegetative forms with ability to grow at higher pH values than could the original culture, rather than to select spores that could germinate at higher pH values (Kushner & Lisson, 1959). It was conceivable that such alkali-resistant vegetative forms might produce spores whose germination was as sensitive to highly alkaline conditions as were spores of the original alkali-sensitive cultures. It has now been found that spores of this selected alkali-resistant strain can germinate and develop into vegetative rods at higher pH values than spores of the original strain Mu-3055. The upper pH limit for spore germination of *B. cereus* strain R spores is slightly lower than the upper limit for growth.

Spores of the alkali-resistant *Bacillus circulans* Ru 38 can also germinate in highly alkaline media; when incubation is at 30°, they can germinate in a medium of the highest pH value at which growth is possible. Germination and outgrowth took place more rapidly at pH 9.5–10.3 than at pH 7.4; and it should be noted that vegetative growth of *B. circulans* Ru 38 began after a shorter lag period at pH 9.9 than it did at pH 7.4 (Chislett & Kushner, 1961). The fact that spores of the bacteria studied had the ability to develop into vegetative forms and these to multiply at high pH values suggests that the alkali resistance of these processes may have a common physiological basis.

In so far as it has been studied, the effect of temperature on the processes of growth and spore germination at different pH values seems complex. Although a temperature of 37° could cause growth to take place more rapidly than a temperature of 30°, at the highest pH values at which germination and growth were possible, germination, outgrowth and vegetative growth of *Bacillus cereus* R and outgrowth of *B. circulans* Ru 38 took place more rapidly, or only at 30°. Thus, despite its ability to accelerate growth, the higher temperature also increased the toxic effects of highly alkaline media.

The alkali-resistant *Bacillus circulans* strain Ru 38 has been deposited with the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland, and allotted the number NCIB 9218.

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The Diminution of Variation in Bacterial Populations with Special Reference to *Klebsiella pneumoniae* and Drug Resistance

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SUMMARY

Single organisms of *Klebsiella pneumoniae* were repeatedly selected for resistance on streptomycin agar yielding a population with increased resistance clearly different from that of the parent strain. These two strains were grown together and when retested a uniform population was found to be present with streptomycin resistance intermediate between the initial two.

Two strains of *Klebsiella pneumoniae*, one the type-strain, the other a chloramphenicol-resistant mutant with a different colonial appearance, were grown together and the mixture sampled at intervals. The two populations became progressively more alike until within six hours they were indistinguishable. The resulting uniform population was intermediate in sensitivity to chloramphenicol and the colonies could no longer be assigned easily to either parental type on morphological grounds. When the two strains were separated by a collodion membrane, this diminution of variation did not occur.

The results cannot be explained by overgrowth of one strain by the other. It is suggested that something analogous to inbreeding is taking place.

INTRODUCTION

Previous work has shown that individual bacteria within clones differ from one another (Hughes, 1952, 1953, 1955*a*, *b*). This type of variation is continuous and more common than mutation as ordinarily understood. This work was summarized by Hughes (1957), and the conclusions reached agree in the main with the view advanced by Yudkin (1953) based on his own studies about acquired drug resistance. The subject has received mathematical consideration from Powell (1956, 1958).

The following work was undertaken to find out why, if variation could be found between the individuals, the population as a whole remained stable. In an organism in which sexual reproduction is recognized this is no problem, since any major variation or mutation which is not lethal will be distributed by random crossing. The predominant views on bacterial reproduction predispose against accepting a similar mechanism among cells which multiply by so-called simple fission.

It has now been found that when two populations, derived from the same source but which are different in regard to resistance to streptomycin, chloramphenicol and other characters, are grown together for a short time, a uniform population inter-

mediate in respect to the characters studied emerges very rapidly. This diminution of variation appears to differ in its general properties from the changes associated with established parasexual mechanisms, such as transduction, transformation or conjugation.

METHODS

Organism. A strain of *Klebsiella pneumoniae* (NCTC 7242) first obtained in 1946 for the assay of streptomycin was used. This had been repeatedly subcultured.

Isolation of single organisms. A De Fonbrune micromanipulator and a cheap modification of his oil chamber (made by Hawksley and Sons Ltd., London) were used throughout this series of experiments.

Experimental procedures. Young cultures were obtained by inoculating one loopful of the stock culture stored on the bench into nutrient broth in a screw-capped bottle. This was clipped to a drum rotating at 8 rev./min. in an incubator at 37° and left for 3 hr. One 3.5 mm. loopful of this subculture was spread on a block of agar about 1.0 cm. square mounted on the cover glass of the oil chamber. After air drying the coverslip was reversed and the oil introduced. The specimen was then incubated, usually for 3 hr., and the size of individual colonies recorded. In the presence of streptomycin or chloramphenicol, the colony diameter is an index of the average resistance of the organisms of the colony to the concentration of antibiotic in the agar. Measurements in arbitrary units were made with an eye-piece micrometer (total magnification usually 240 diameters); 100 colonies were measured and the histogram of the population plotted. Any colonies which had fused were discarded from the count.

The planting out of single organisms was impracticable since the time lag between the first and the last isolations would invalidate the investigation. On special occasions the specimens were examined when only one or two divisions had taken place and any colonies found to be too near to one another were decreased in number by removing those likely to come into contact. This was not usually necessary since, with the small implants used less than 5% of the colonies would have been involved and these could have been recognized and excluded after incubation.

From the most rapidly growing colonies single organisms were picked and grown on to give strains which were again tested for their rates of growth at the same concentration of antibiotic. Those found to be growing better than the previous strains were retained as having a higher resistance. This sequence was repeated until a strain had been selected which grew as well on antibiotic agar as the parent strain on plain nutrient agar. It had already been demonstrated that single streptomycin-resistant cells of *Klebsiella pneumoniae* could be selected and stable strains obtained (Hughes, 1957) as had been done previously with staphylococci with regard to penicillin (Hughes, 1952; Eagle, Fleischman & Levy, 1952).

Having obtained two strains which could be separated by the rates of growth of their colonies in the presence of antibiotic it became possible to reconsider Yudkin's hypothesis on populations of bacteria (Yudkin, 1953). Clearly if differences between organisms could be demonstrated in small populations, then in large populations a great diversity should occur; experience does not support this and there must be some mechanism to counter excessive variation.

RESULTS

Experiment 1 was designed to demonstrate whether or not the two strains grown together would influence one another's resistance to antibiotic. A loopful of a 3 hr. broth culture of the original strain and a similar inoculum of the 17th single cell subculture (each of which had been selected for increased resistance) were separately planted on 1.0 cm. square blocks cut from a poured nutrient agar plate containing 10 $\mu\text{g./ml.}$ of streptomycin. An equal volume of a 17 hr. mixed culture of the two strains diluted to the equivalent concentration was similarly seeded. The three blocks were arranged in the same oil chamber, covered with sterile paraffin oil and incubated at 37° for 3 hr., when the colony diameters were measured.

The resulting histograms are shown in Fig. 1; after incubation together in mixed culture instead of the two peaks characterizing the separate cultures, only a uniform population was present, the average colony diameter falling between the two extremes but, on an arithmetic scale, nearer to the size of the 'sensitive' colony. This strongly suggested that the two populations had, if not 'interbred', at least influenced one another, and further study of the phenomenon was made.

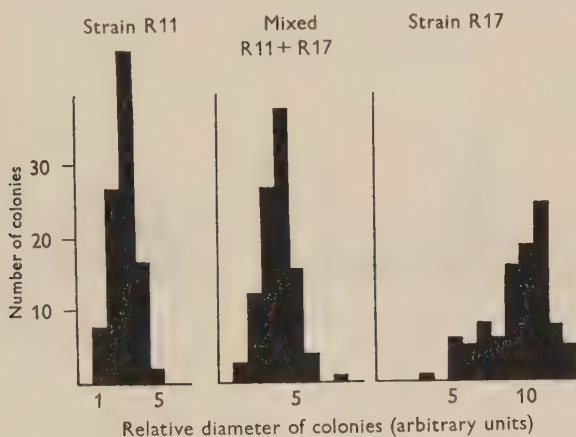


Fig. 1. Histograms showing distribution of size among colonies, growing for 3 hr. on agar containing streptomycin (10 $\mu\text{g./ml.}$), derived from a streptomycin-sensitive strain of *Klebsiella pneumoniae* NCTC 7242 (R11) and a more resistant strain derived from it (R17). Colony diameter measured in arbitrary units.

Experiment 2. Certain difficulties had been apparent in the earlier experimental arrangements. For example, with streptomycin it was only possible to select for resistance, not for sensitivity, since streptomycin is bactericidal and the most susceptible members of all the populations were lost. Further, streptomycin was unstable under the conditions in which the agar blocks were prepared. In all subsequent experiments freshly prepared chloramphenicol solutions were used.

Into a dense suspension of *Klebsiella pneumoniae* an equal volume of broth containing chloramphenicol was poured so that the final concentration of chloramphenicol was near the inhibitory concentration for small implants, in this case 8 units/ml. The mixture was incubated overnight and then planted as a wash on a ditch plate containing 30 units chloramphenicol in the ditch of a Petri dish con-

taining 15 ml. agar medium. One single colony was found near the ditch. The strain so obtained (KR) was compared with one of the sensitive strains (K5) chosen for its neat colony form and reliable cultural characteristics. A mixed culture of the two strains in broth at 37°, was sampled at intervals, diluted in broth and planted on agar blocks containing chloramphenicol (8 units/ml.). The filled oil chambers were cooled at 4° until the full series had been collected. They were incubated overnight (17 hr. usually) at 20–22° and then refrigerated again to stop further growth, before being measured.

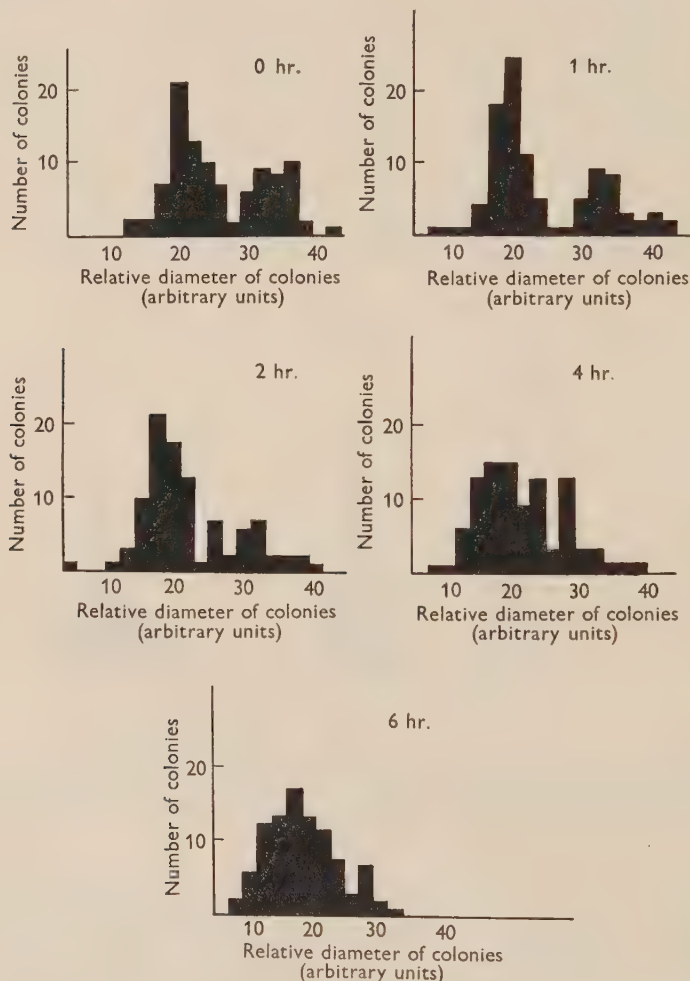


Fig. 2. Histograms showing distribution of size among colonies, growing overnight at 20–22° on agar containing chloramphenicol (8 units/ml), derived from a mixed culture of a sensitive (K5) and a resistant (KR) strain of *Klebsiella pneumoniae*. Samples were plated at 0, 1, 2, 4 and 6 hr. after mixing.

The histograms in Fig. 2 make clear the transition from two independent populations to one which was uniform. The whole cycle with a stationary culture appeared to be complete in only 6 hr. It was fortunate that the resistant strain here used was

distinguishable from the sensitive strain. The resistant strain produced much more slime and capsular material than did the sensitive strain; broth cultures were sticky and tenacious when touched with a wire loop. The individual colonies of the resistant strain at all stages of growth were recognizably different from the sensitive colonies. The resistant colonies were irregular in outline with a coarser surface texture, whereas the sensitive were circular and uniformly smooth.

During incubation with the sensitive strain the size of the colonies of the resistant strain in the samples became progressively smaller and many were morphologically intermediate in type so that while at first it was easily possible to score the two strains separately, later the characters were so mixed as to make this difficult. In Fig. 2, as in other diagrams, not all of the results available are shown; the readings at 3, 5, 7 and 8 hr., however, are in complete accord. A number of controls had to be set up before it could be stated that the change of size, that is the alteration of resistance, was due to the influence of the one strain on the other.

Experiment 3. In order to discover whether the change in average size of colony was due to an overgrowing of one strain by the other in broth culture, the opacity of growing broth cultures of the two strains, separately and together, was measured with a Hilger-Watts Spekker absorptiometer.

The results are shown in Fig. 3; the resistant strain evidently had a longer lag period. When an old culture was used there would therefore be a delay in growth as compared with the sensitive strain, and this would diminish the differences between the two. This difficulty was overcome when cultures already grown in broth for 3 hr. were used.

Experiment 4. To find whether the conditions for growth in the oil chamber favoured one strain more than the other the two were grown both separately and together on plain agar. Under these conditions the sensitive strain formed the larger colonies but both grew well. When equal volumes of young cultures of the two strains were planted together in plain broth and sampled at intervals on to agar, it was found that the ratio of the one to the other did not alter appreciably during incubation. The strains could be differentiated from one another by the colony morphology, at least during the first 6 hr. of incubation. This observation agrees well with the results of the opacity readings in Fig. 3.

Experiment 5. To show whether actual contact is needed between the cells of the two strains in order to bring about the changes seen when they are grown together, the standard experiment was carried out with the two strains separated from each other by a collodion (Gradocol) membrane of average pore size 0.46μ . The resulting histograms do not suggest that there was any significant change (Fig. 4).

Experiment 6. It was decided to investigate the effect of disturbance on the cultures. The mixture of organisms was put up as before but in tubes which were rocked in a water bath at 37° with about 45 oscillations/min. Samples were taken as before. The preparations were inspected to exclude any possible aggregation into pairs, so that the colonies measured should not be the product of conjoined pairs of organisms. The results are shown in Fig. 5. Again it will be seen that the same trend was present, the shaking merely accelerated the result. A technical difficulty that has not yet been overcome may be mentioned here. Materials for an experiment are prepared and the organisms planted and incubated in broth. Samples withdrawn at predetermined intervals are transferred to the agar medium in oil chambers and

stored in the refrigerator. The results obtained on subsequent culture depend, if they are to be compared, on the starting temperature of each oil chamber being identical and on all the organisms remaining equally in the log phase of growth. When sampling is at hourly intervals, then the rate of equilibration is important; the 7 hr. specimen will be exposed for only 30 min. at 4° while the earlier ones will have had 1.5, 2.5 hr., respectively, and so on. It appears from our results that something of

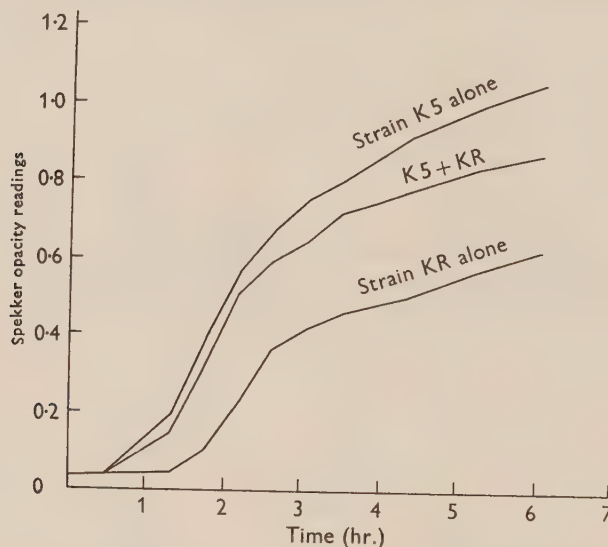


Fig. 3. Growth curves of sensitive strain (K5) and resistant mutant (KR) of *Klebsiella pneumoniae*, in separate and mixed broth culture at 37°. Opacity readings were made with a Spekker absorptiometer.

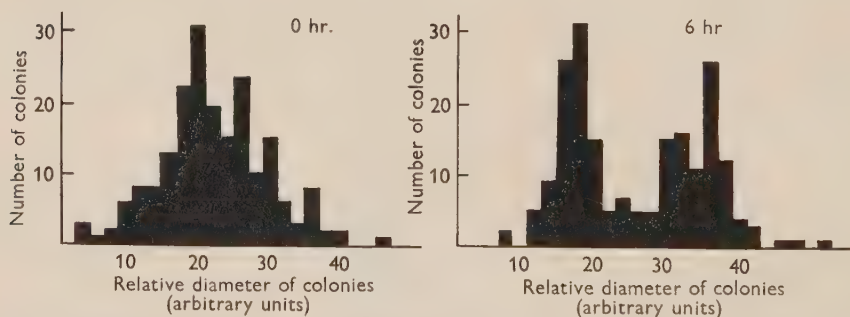


Fig. 4. Histograms showing the distribution of the size of colonies of the sensitive (K5) and resistant (KR) strains of *Klebsiella pneumoniae*, grown in broth and separated from each other by a Gradocol membrane.

the order of cooling for 3 hr. is desirable in the English summer. This conclusion was based on the following observation. A micromanipulator cell, filled with oil containing the agar block was connected to a thermocouple inside a refrigerator, the galvanometer being on a support outside. It was found that while cooling from room temperature began rapidly and evenly, at the lower range temperature fluctuations were marked and a settled low value was not reached until at least 3 hr. By

this time the organisms of the early samples would have passed into the lag phase and all late samples in the series will appear to grow more rapidly than the earlier. This might be overcome by refrigerating for a prolonged period when all samples would show the same lag.

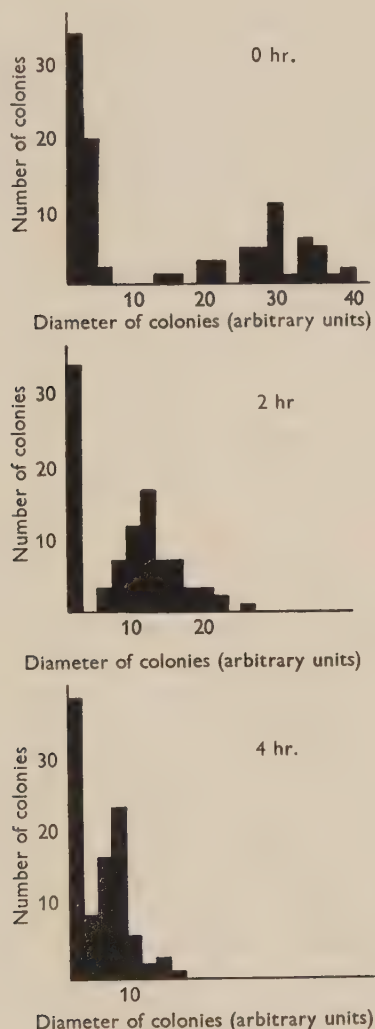


Fig. 5. Histograms showing the distribution of colony size as before. Sensitive and resistant strains of *Klebsiella pneumoniae* grown together in a broth culture, rocked at about 45 oscillations/min. in a water bath at 37°, and sampled on the 8 units chloramphenicol/ml. agar.

DISCUSSION

It has long been recognized that many drug-resistant 'mutants' selected by whatever method tend to alter on subculture, becoming rather more sensitive than when first isolated. This can be explained easily enough if it be supposed that the colony taken contains a mixture of organisms which, when in subculture, influence one

another towards a mean. In the present experiments two strains both derived from the same pure culture of *Klebsiella pneumoniae* and grown repeatedly from single organisms, when incubated together merged progressively into a uniform population. This disappearance of the mutant strain or, in the first series of experiments where streptomycin was used, of the clone selected for streptomycin-resistance, was not due to overgrowth by the sensitive strain, for, when the growth rates were balanced by using only actively growing cultures, the ratio of the numbers of one strain to the other remained constant throughout the experiment.

That the strains did not influence one another when separated by a coarse grade of collodion membrane through which any large molecule would pass easily suggests that actual contact between the organisms may be necessary. Relatively gentle agitation only accelerated the process and did not at all inhibit it. A suitable technique for demonstrating the results of violent agitation such as is needed to separate recombining *Escherichia coli* organisms (Wollman, Jacob & Hayes, 1956) has not been devised for the present work.

A demonstration of the kind given here seems necessary as a corollary to Yudkin's work and in particular to his theoretical considerations about bacterial populations and the emergence of resistant strains. Yudkin pointed out (1953; Fig. 2) that if cells divided 'unequally' then a population would be obtained which would be progressively variable when grown in the absence of a drug (or of other selecting mechanism) until, after many subcultures, the permanent form of the distribution curve would be reached. For one character, e.g. drug resistance, there is obviously a limit to variation; finality is reached when the population contains some completely resistant individuals. For all the factors that make up the complex genetic pattern of the population, almost infinite variation is possible. Nevertheless, since various microbial diseases described by ancient writers are still recognizable today, their causal organisms cannot have greatly changed. Similarly, when the Oxford staphylococcus was collected from a number of centres and re-examined after about 10 years' use as a standard, variation was within the limits of the assay methods in use. This is no longer true, however; it is probable that substitution of other strains has now taken place (Oeding & Ostervold, 1959).

It seems probable that the relative uniformity of members of microbial populations depends on regular interchange of hereditary material. Under the highly artificial conditions of laboratory culture on solid media, or where deliberate selection with chemicals or otherwise takes place, pressures will be brought to bear on the population which will disclose its latent heterogeneity. Usually variations can be detected only in early divisions of the organisms on solid medium; once they have been separated as isolated colonies, no further variation takes place. This was noticed in the earlier experiments with spontaneously occurring long forms of *Escherichia coli* (Hughes, 1953). Progressive homogenization of a mixture of strains has been found repeatedly, irrespective of the time of incubation or the concentration of antibiotic used for the detection of strain differences.

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CORRIGENDUM

IN BUTTERY, S. H. & PLACKETT, P. (1960). *J. gen. Microbiol.* **23**, 357–368.

On p. 361, lines 17–18:

for: ‘an equal volume of a phenol + water mixture (1:1)’

read: ‘an equal volume of a phenol + water mixture (6:1, w/w)’

The Mucopeptides of Bacterial Cell Walls. A Review

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The cell walls are rigid insoluble structures of bacteria. Attempts at isolating walls were first made last century (Vincenzi, 1887), and there was much speculation about their chemical nature—chiefly as to whether they consisted of chitin or cellulose, materials known to provide rigid structures in other organisms. Even 10 years ago practically nothing could be said on the subject except that the walls were not chitin or cellulose. Since then we have achieved at least the beginning of an answer (see Reviews, Salton, 1956*a*; Work, 1957; Zilliken, 1959); but it will be long before we have the full story for all types of bacteria. The first information about the chemical constituents of bacterial walls was in a report by Holdsworth (1951) that phenol-insoluble residues from *Corynebacterium diphtheriae* contained nearly all the glucosamine and α, ϵ -diaminopimelic acid of the organism, and that these compounds were associated with a polysaccharide. The perfection of techniques for preparation of walls (Salton & Horne, 1951), by mechanical disintegration and differential centrifugation, enabled apparently homogeneous samples to be examined. Walls of streptococci were shown simultaneously by Salton (1952*a*) and McCarty (1952) to contain about 70 % of polysaccharides and hexosamines and a further component consisting of only 10 amino acids, of which lysine, alanine and glutamic acid preponderated. Rhamnose was the main polysaccharide; in fact, over 90 % of the total rhamnose of the cell was in the wall. Further work by Salton showed that walls of other species also had an unusual composition, which was not then understood.

The problem was attacked systematically by Cummins & Harris (1956*a, b*, 1958; Cummins, 1956) who examined walls of over a hundred Gram-positive bacteria. The separated walls were treated with trypsin and ribonuclease, and were thus freed from wall proteins and cytoplasmic constituents. A resistant residue was left; its main components, identified by paper chromatography of acid-hydrolysates, were invariably the two hexosamines glucosamine and muramic acid, and three amino acids, glutamic acid, alanine and either lysine or diaminopimelic acid; in some cases there were also up to five sugars, one or two other amino acids, or galactosamine. A recurring type of 'basal unit' in Gram-positive cell walls was soon recognized (Work, 1957). This 'basal unit' can be termed a 'mucopeptide'; evidence for its existence will be presented in this review. It contains the aforementioned hexosamines, glucosamine and muramic acid, and three amino acids bound in peptide linkage. In addition, each bacterial genus and even each species often has a characteristic pattern of amino acids, amino sugars and sugars superimposed on the basal mucopeptide unit. This pattern may prove of value in bacterial classification.

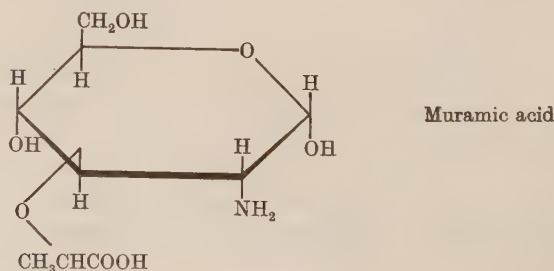
The basal unit was apparent from the results of Cummins & Harris, because they had, so to speak, stripped the meat off the walls with enzymes, leaving only the rigid framework. However, these results give an over-simplified idea of cell-wall structure, and it should be realized that freshly separated walls of Gram-positive organisms may contain up to 40 % of their weight of protein and other compounds. For example, the teichoic acids, which are polyribityl phosphates, were found by Armstrong *et al.* (1958) to make up as much as 30 % of the walls of certain Gram-positive organisms. The walls or 'coats' of bacterial spores also contain mucopeptide units with considerable proportions of proteins and other unidentified substances (Strange & Powell, 1954; Strange & Dark, 1956; Salton & Marshall, 1959).

The walls of Gram-negative bacteria are more complex than those of Gram-positive bacteria; in addition to polysaccharide, the trypsin-treated walls contain a high proportion of lipid and most of the amino acids found in proteins (Salton, 1956*a*). The specific mucopeptide constituents, glucosamine, muramic acid and diaminopimelic acid, are also present, but their overall concentrations are lower than in the walls of Gram-positive organisms. The walls of *Escherichia coli* appear to have three layers visible with the electron microscope (Kellenberger & Ryter, 1958). Chemically, they can be separated into an outer pliable lipoprotein coat very soluble in phenol, and an inner insoluble rigid layer containing mucopeptide constituents; a further less soluble constituent is a lipopolysaccharide which is probably the site of the O-antigen (endotoxin) (Weidel & Primosigh, 1958; Weidel, Frank & Martin, 1960; Westphal, 1960). The mucopeptide components are different from those of walls of Gram-positive bacteria in that they contain both diaminopimelic acid and lysine.

The rigidity and insolubility of at least part of the walls of nearly all types of bacteria suggest that the mucopeptide is highly polymerized and perhaps highly cross-linked. Owing to this insolubility, the chemical homogeneity of the rigid portions of wall preparations cannot be assessed, nor can chemical information on their undegraded structures be obtained directly. However, it is possible to purify soluble subunits of the rigid portions, and their analysis has produced evidence for the mucopeptide structures.

Structure and metabolism of some specific constituents of mucopeptides

Several constituents of bacterial mucopeptides have not been found elsewhere in nature, and may be specific to these structures. Muramic acid was isolated by Strange (1956) from a product obtained from the exudates of germinating spores of *Bacillus megaterium*; it is glucosamine in ether linkage through its 3-position with lactic acid (Strange & Kent, 1959):



Muramic acid is a component of walls of all bacteria, but has not yet been identified in other types of organisms. It probably originates from glucosamine (Zillikin, 1959; Richmond & Perkins, 1960*c*).

Whenever the optical configuration of the amino acids in Gram-positive cell walls has been examined, almost all the glutamic acid and at least 50 % of the alanine have been identified as the so-called 'unnatural' D-isomers (Ikawa & Snell, 1960; Salton, 1957*b*). D-aspartic acid has also been found (Toennies, Bakay & Shockman, 1959), but never D-lysine. Rydon (1948) stated that D-amino acids are certainly present in bacterial extracellular products such as antibiotics or capsular polyglutamic acids, but that they had not been encountered in the intracellular components of micro-organisms. Now, only 12 years later, we know that certain D-amino acids are major components of many bacterial walls, which themselves make up 25 % or more of the total dry weight of the organisms. The origin of these D-amino acids can be traced partly to the action of alanine racemase, a widely distributed bacterial enzyme. In *Bacillus* species, D-glutamic acid originates from L-glutamic by a series of reactions. A transamination between L-glutamic and pyruvic acids gives L-alanine, which is then racemized to the racemic mixture; of this, the D-alanine transaminates with α -oxoglutarate to form D-glutamic acid and pyruvic acid (Thorne, Gomez & Housewright, 1955; Thorne, 1956). The transaminase responsible for the last reaction is stereospecific, and will only carry out the reverse step with D-glutamic or D-aspartic acids, the L-isomers being inactive. A glutamic acid racemase has recently been isolated from *Lactobacillus arabinosus* (Glaser, 1960), but its significance in wall metabolism is unknown.

α,ϵ -Diaminopimelic acid is another compound specific to bacteria (see Review by Rhuland, 1960). It is not found in any other micro-organisms with the exception of the blue-green algae (Myxophyceae) which are closely related to bacteria. It is present in all bacteria, with the exception of most Gram-positive cocci and various lactobacilli (Work, 1951; Work & Dewey, 1953; Hoare & Work, 1957). Chemically

diaminopimelic acid $\begin{array}{c} \text{COOH} \\ | \\ \text{NH}_2 \end{array} \text{CH} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH} \begin{array}{c} \text{COOH} \\ | \\ \text{NH}_2 \end{array}$ is unlike most other naturally occurring amino acids in that it has 2 amino and 2 carboxyl groups. It has 3 stereoisomers, LL, DD, and one *meso* (DL) form, the molecule being symmetrical about the γ -carbon atom. The form which was originally isolated from *Corynebacterium diphtheriae* was optically inactive and was proved to be the *meso* isomer (Work *et al.* 1955). A solvent which will separate the *meso* isomer from the other two on paper chromatograms has enabled the isomer present in any organism to be determined (Rhuland *et al.* 1955; Hoare & Work, 1955). The *meso* form is that most commonly found, but in certain families, notably Propionibacteria, and many Streptomyces, the LL isomer is present; a few organisms contain both *meso* and LL forms (Hoare & Work, 1955, 1957). The findings of Cummins & Harris and of Salton on distribution and isomeric form of diaminopimelic acid in cell walls of various species are identical with our results on whole bacteria. In fact, the majority of the cellular diaminopimelic acid is located in the wall, although small amounts are sometimes found in soluble fractions.

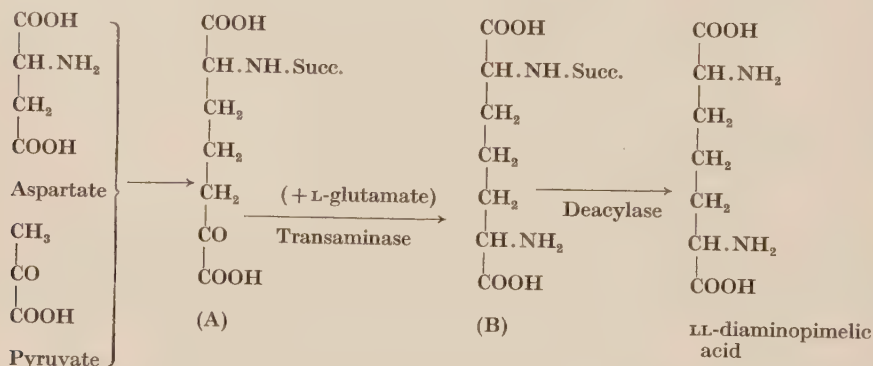
Diaminopimelic acid undergoes the general types of metabolic reactions which occur with other amino acids. The *meso* and LL isomers are attacked by certain L-amino acid oxidases (Work, 1955); some more specific bacterial enzymes decarboxylate, racemize, oxidize or transaminate one or other isomer (Dewey & Work,

1952; Dewey, Hoare & Work, 1954; Antia, Hoare & Work, 1957; Meadow & Work, 1958; Antia & Work, 1961). The racemase and decarboxylase have been studied in detail.



Diaminopimelic racemase converts either the *meso* or LL isomer to an equilibrium mixture of the two, while the decarboxylase converts *meso*-diaminopimelic acid to L-lysine with evolution of 1 molecule of CO₂. Thus, *meso*-diaminopimelic acid is attacked by these two enzymes at the D 'end'; this is the only known enzymic decarboxylation of a carboxyl group in the D-configuration. Since the DD isomer is not attacked by either enzyme, the L-configuration at one end of the molecule is evidently essential for fixation to the enzymes.

The bisynthesis of diaminopimelic acid with cell-free preparations of various *Escherichia coli* mutants has been partly worked out (Gilvarg, 1957, 1958, 1959; Kindler & Gilvarg, 1960; Peterkofsky & Gilvarg, 1959).



Some of these preparations can synthesize the amino acid from aspartic acid and pyruvic acid in the presence of glutamate, ATP and various cofactors. A monoketo-monoamino acid (compound A) was identified as an intermediate: the amino group was derived directly from that of aspartate and was acylated with a succinyl radical. The acylation protects the molecule from cyclization, which would undoubtedly occur if the amino group were free to react with the keto group. A transamination with L-glutamate as specific amino donor results in the formation of *N*-succinyl-L-diaminopimelic acid (compound B), which is converted to LL-diaminopimelate by a specific deacylase. Since *E. coli* contains *meso*-diaminopimelic acid, the next step is probably racemization of the LL isomer. There are several lines of evidence that this step occurs in the whole organism (Hoare & Work, 1955; Meadow, Hoare & Work, 1957), and a metabolic function for diaminopimelic racemase has been suggested.

The function of diaminopimelic decarboxylase is the formation of lysine. This was first established when it was found that certain mutants of *Escherichia coli* which require lysine lacked the decarboxylase (Dewey & Work, 1952). Formal proof of *in vivo* conversion of diaminopimelic to lysine was obtained by examining the

incorporation of radioactive diaminopimelic acid into the amino acids of growing cultures of *E. coli* (Meadow & Work, 1959). It was possible to trace the radioactivity from diaminopimelic acid to lysine, as excellent recovery of exogenous radioactive lysine was obtained in the cellular lysine. Also, radioautography showed that radioactive diaminopimelic acid was converted only into radioactive lysine, and that neither amino acid interchanged its carbon chain with other amino acids. To determine the proportion of lysine originating from diaminopimelic acid, two mutants of *E. coli* were studied: one (173-25), had a relative requirement for lysine and an absolute requirement for diaminopimelic acid (Davis, 1952); the other (D), was derived from mutant 173-25 by 'training' it to dispense with lysine, when it required to be given only diaminopimelic acid (Meadow *et al.* 1957). The mutants

Table 1. Summary of ^{14}C -incorporation experiments with diaminopimelic acid (DAP)-requiring mutants of *Escherichia coli*

The organisms were grown in minimal media containing glucose (0.5%), lysine (0.1 mM) and DAP (0.1 mM). In each experiment one of these sources of carbon was radioactive (totally labelled). Phenol-insoluble fractions of walls were hydrolysed and the radioactivity of amino acids determined (Meadow & Work, 1959).

Mutant 173-25 requires to be given lysine and DAP; mutant D requires only DAP.

Expt.	^{14}C source	Mutant	Radioactivity of wall amino acids			Radioactive lysine as % of total lysine of fraction
			DAP	Lysine	Others	
1	DAP	173-25	+	+	0	80
		D	+	+	0	55
2	Lysine	173-25	0	+	0	10
		D	0	+	0	10
3	Glucose	173-25	0	+	+	10
		D	0	+	+	20

were grown in a basal salts and glucose medium with lysine and diaminopimelic acid, but in each experiment either the glucose, lysine or diaminopimelic acid was totally labelled with ^{14}C . The cell walls were fractionated with phenol, and the insoluble portions, containing most of the mucopeptide, were hydrolysed and the proportion of the lysine originating from each carbon source was calculated (Table 1). Expt. 1 showed that labelled diaminopimelic acid was converted to lysine, but that it accounted for 80 % of the total wall lysine in mutant 173-25 which required to be given lysine for full growth, and only 55 % in mutant D. This difference between the two mutants was not accounted for by differences in their utilization of exogenous lysine, since they incorporated identical amounts of radioactivity from ^{14}C -lysine (Expt. 2). When labelled glucose was used (Expt. 3), all the amino acids except diaminopimelic were labelled, and the mutant with no lysine requirement incorporated twice as much radioactivity into lysine as the other mutant. Expt. 3 proved that part of the lysine was derived from a route other than through diaminopimelic acid, and that there were at least two biosynthetic paths to lysine in *E. coli*. The major route is through diaminopimelic acid, while the other utilizes some unknown carbon source. The latter route was evidently strengthened by 'training' the mutant to dispense with added lysine, since the diaminopimelic decarboxylase activity was not changed by the process (Meadow *et al.* 1957).

Bacteriolytic enzymes

Although the mucopeptides of bacterial cell walls are resistant to proteolytic enzymes (perhaps because of the presence of D-amino acids), they are often very susceptible to attack by bacteriolytic enzymes. The best known is lysozyme, first shown by Fleming (1922) to lyse living cultures of *Micrococcus lysodeikticus*, and later found to attack certain other living Gram-positive bacteria. Salton (1952*b*) showed that the substrate for lysozyme is the cell wall itself; wall preparations from susceptible species are completely solubilized by lysozyme treatment, with a simultaneous decrease in optical density and the liberation of substances giving reactions for reducing sugars and *N*-acetylhexosamines (Reviews, Salton, 1957*a*, 1958).

A so-called disaccharide has been identified in some lysozyme digests (Salton, 1956*b*). It is probably a $\beta(1 \rightarrow 6)$ glycoside of *N*-acetylglucosamine and *N*-acetylmuramic acid, and is responsible for the reducing action of the digests. The reducing group belongs to muramic acid (Salton & Ghuysen, 1959; Perkins, 1960*a*) so that the 1-carbon of glucosamine may be linked to the 6-carbon of muramic acid. Small amounts of a lysozyme-sensitive tetrasaccharide also occur in the digests; this may be a dimer of the disaccharide joined by $\beta(1 \rightarrow 4)$ linkages. Since free *N*-acetylglucosamine or *N*-acetylmuramic acid have not been found in digests, it seems possible that at least part of the mucopeptide molecule contains these compounds bound in pairs by lysozyme-sensitive $\beta(1 \rightarrow 4)$ linkages alternating with lysozyme-resistant $\beta(1 \rightarrow 6)$ linkages; they might, in fact, form part of a backbone to which are linked peptides and polysaccharides. If this theory is correct, the $\beta(1 \rightarrow 4)$ glycosidic linkages in the backbone are the substrate for lysozyme. The susceptible links may be unevenly distributed along the backbone, since the fragments are of very different sizes and composition, some even have molecular weights up to 15,000, and contain sugars, amino acids, muramic acid and glucosamine. There are great differences in susceptibility to lysozyme among various bacterial species. Little is known about the exact structure in walls which determines the extent of lysozyme sensitivity; no correlation has been found between overall composition and this sensitivity. All we know is that certain lysozyme-resistant organisms contain more O-acetyl groups in their walls than do lysozyme-sensitive strains of the same organisms, and that the removal of these groups with mild alkali often renders the cells more sensitive (Brumfitt, 1959).

Most living Gram-negative bacteria are resistant to lysozyme, but they can be rendered susceptible by subjection to extremes of temperature or pH or by exposure to surface-active agents such as detergents, bile salts, chloroform, polymyxin or ethylene diamine tetra-acetic acid (EDTA) (see Review, Salton, 1958). These treatments probably all cause damage to the outer lipoprotein coat, and thus allow access of lysozyme to the inner mucopeptide substrate. Wall suspensions from Gram-negative bacteria when treated with lysozyme, show no appreciable changes in optical density, probably owing to their high content of non-mucopeptide constituents. Soluble constituents are, however, liberated from the walls; some contain diaminopimelic acid, alanine, glutamic acid, glucosamine and muramic acid (Salton, 1958; Work & Lecadet, 1960).

Bacteriolytic enzymes are also produced by some bacteria. Many of these enzymes resemble lysozyme in their substrate specificity and in the nature of their

digestion products (Richmond, 1959*a, b*); but there are often small differences of specificity and point of attack between the different enzymes, which are not yet understood. The bacteriolytic enzymes which do not resemble lysozyme were reviewed by Work (1957) and by Strange (1959). These enzymes have been found in the culture filtrates of certain *Streptomyces* species or associated with spores and vegetative cells of aerobic sporulating species of the genus *Bacillus*. The enzymes from spores are not entirely species-specific, although they are often more active towards walls of their own species; they will even attack walls of Gram-negative bacteria after treatments similar to those used for sensitization to lysozyme (Work, 1959). It has been suggested (Strange, 1959) that the lytic enzymes of species of *Bacillus* are involved in the sporulation cycle, since they are most active in the sporulating and germinating phases, when they release soluble mucopeptides from the sporangial wall or spore coat (Powell & Strange, 1956).

The main mucopeptide released from germinating spores of *Bacillus* species has been isolated (Powell & Strange, 1953; Strange & Powell, 1954). It has a molecular weight of about 15,000 and consists mostly of 1 molecular proportion of D-glutamic acid, 3 of alanine (D and L isomers), and 1 of *meso*-diaminopimelic acid, and a mixture of 8 molecular proportions of acetylglucosamine and acetylmuramic acid (Strange, 1959). This mucopeptide served as the original source of muramic acid. The mucopeptide is the only high-molecular-weight degradation product of wall mucopeptides to have been purified and analysed; the sequence of amino acids in it has not yet been established. It is further degraded by lysozyme and by β -glucosaminidase. Both glucosamine and muramic acid are present as the *N*-acetyl derivatives. This is the case with all the enzymic degradation products of walls so far examined (Salton, 1956*b*; Salton & Ghuysen, 1959; Perkins, 1960*a, b*; Ghuysen & Salton, 1960) and it may be assumed that the amino groups of these hexosamines are always acetylated.

Other lytic enzymes of bacteria are associated with the cell walls of vegetative organisms (Mitchell & Moyle, 1957), and may be responsible for the rapid autolysis which sometimes occurs in dense suspensions of these organisms. Autolytic activity, if present, is stronger in rapidly growing cultures, and may play an active part in cell division, since localized breakdown of rigid cell-wall material might well occur immediately before division, followed by immediate resynthesis.

Lysis by bacteriophages. Lytic enzymes which attack cell walls are also associated with bacteriophages (Panijel & Huppert, 1957; Koch & Weidel, 1956; Maxted, 1957; Murphy, 1957; Koch & Jordan, 1957; Brown & Kozloff, 1957; Ralston, Lieberman, Baer & Krueger, 1957; Krause, 1958; Koch & Dreyer, 1958; Weidel & Primosigh, 1957, 1958). Probably these enzymes are localized in the phage tails and are responsible both for the penetration of the phage infective principle through the bacterial rigid wall layer, and for the subsequent lysis of infected cultures (see review by Weidel, 1958). The lysates themselves often also show lytic activity. A defective lysogenic mutant (P_{32}) of *Escherichia coli* K₁₂(λ) produces a lysate which contains a lytic enzyme (Jacob & Fuerst, 1958). Some of the properties of this enzyme (known as λ -endolysin) have been investigated after partial purification from a lysate induced by nitrogen mustard (Work, 1960). λ -Endolysin does not attack viable bacteria, even the host strain of *E. coli*, except after certain preliminary treatments, e.g. with chloroform, EDTA, acetone-drying. The bacteria

are only susceptible during the exponential phase of growth. Many Gram-negative species are attacked, and even some Gram-positive species (*Bacillus megaterium*, *Staphylococcus aureus*). *Micrococcus lysodeikticus*, the substrate *par excellence* for lysozyme is not susceptible to λ -endolysin. Cell walls of *B. megaterium* are completely lysed by λ -endolysin, and have been used as substrate to examine its properties. The optimum pH value is about 7.0; some activation is produced by NaCl or KCl (10^{-2} M). Phosphates and certain heavy-metal ions are strongly inhibitory, the amounts which give 50% inhibition (in presence of NaCl) being KH_2PO_4 , 10^{-3} M; Zn, 5×10^{-4} M; Fe^{+++} , 10^{-5} M; Co^{++} and Mn^{++} , 10^{-3} M no inhibitions, Co^{++} stimulates slightly. In the complete absence of heavy metals, EDTA is without effect but was usually added to walls before treatment with the endolysin as a protection against inhibitory metal ions. λ -Endolysin resembles the lytic enzyme of disrupted mature phage λ (Fisher, 1959), but it differs from lysozyme in its sensitivity to metals and phosphates and in its specificity. Thus, λ -endolysin is distinct from the lytic enzyme of T₂ coli phage or of lysates produced by the phage; this enzyme has been reported to resemble lysozyme (Koch & Dreyer, 1958).

To provide further grounds for the comparison of λ -endolysin with lysozyme, the products of digestion by these enzymes of walls of *Escherichia coli* B and *Bacillus megaterium* were examined (Work & Lecadet, 1960). The soluble fragments were separated by dialysis into two fractions, each of which was treated with 1-fluoro-2:4-dinitrobenzene and then subjected to paper chromatography or paper electrophoresis. The resulting yellow spots were eluted, hydrolysed and examined qualitatively for free and dinitrophenylated amino acids. The hexosamines appeared to be partially destroyed during the hydrolysis of the dinitrophenylated fragments, so that no conclusions could be drawn about their presence. However, in nearly all cases, glucosamine and muramic acid (one or both) were detected in the hydrolysates, as well as a limited number of amino acids, so most of the yellow fragments were probably mucopeptide in nature.

The fragments produced by λ -endolysin from *Bacillus megaterium* were indistinguishable from those produced by lysozyme. Five spots were obtained by paper chromatography (butanol-acetic acid solvent) from each of the dialysable digests (Table 2). The slower-moving spots contained mainly alanine and glutamic acid and had no identifiable end-amino groups; the more mobile spots were larger and also contained dinitrophenyl-alanine and mono-dinitrophenyl-diaminopimelic acid. In the non-dialysable fractions, which were separated by paper electrophoresis at pH 3.6, similar distributions of amino acids occurred in the various spots, except that here diaminopimelic acid was present with both amino groups bound as well as with one attached to dinitrophenol.

λ -Endolysin and lysozyme did not produce identical fragments from *Escherichia coli* walls (Table 3). The digests contained a variety of amino acids, including both lysine and diaminopimelic acid, also glycine, serine and aspartic acid in addition to alanine and glutamic acid. In the dialysable fraction, only glutamic acid was invariably present in all spots; in fact in one spot from the endolysin digest, it was the only major amino acid detected. None of the less mobile spots contained alanine; diaminopimelic acid and lysine were fairly randomly distributed, but only occurred together in spots which were obviously inhomogeneous (e.g. the immobile or most mobile ones). One spot from the endolysin digest contained very large amounts of

alanine, while a neighbouring spot from the lysozyme digest contained nearly all the usual amino acids of a protein but no diaminopimelic acid. A colourless dialysable fragment in digests from both organisms was identified on paper chromatograms by the acetylhexosamine reaction; it was probably the dimer of *N*-acetylglucosamine and *N*-acetylmuramic acid studied by Salton (1956*b*) in lysozyme digests from Gram-positive organisms. In the non-dialysable fractions of *E. coli*, the differences between the actions of the enzymes were not so marked as in the dialysable fractions, but they were apparent. Several spots contained both diaminopimelic acid and lysine, with either both or one of their amino groups bound, or with both free. Further purification of the material from these spots has not yet been done.

Table 2. *Amino acid contents of some peptides in digests of walls of Bacillus megaterium, produced by λ -endolysin (of Escherichia coli K₁₂(λ) lysogenic mutant P₃₂) or lysozyme*

Identical samples of walls (2 mg.) were digested with each enzyme under optimal conditions. The digests were separated by dialysis, and each fraction was dinitrophenylated with fluorodinitrobenzene. The dialysable fractions were spread on Arche 310 paper and irrigated with butanol + acetic acid + water (4:1:1) solvent for 2 days; the non-dialysable fractions were subjected to paper electrophoresis on Whatman 3MM paper at pH 3.6, 500 V for 9 hr. After drying the papers, the yellow spots were each cut out, eluted, hydrolysed with 6*N*-HCl and examined qualitatively by paper chromatography for free and dinitrophenylated amino acids. Controls without walls were treated identically; they showed small amounts of a few amino acids (originating from the filter paper) which have been allowed for in presenting the results.

The figures in the columns represent the distances (cm.) travelled by each spot. The two enzymes produced fragments identical in mobility and amino acid contents.

Dialysable fraction		Non-dialysable fraction	
cm.	Amino acids	cm.	Amino acids
2 → 5.5	Glu, Ala	-1 → -8	Glu, Ala
6 → 12	Glu, Ala	0 → +11	Dap, Glu, Ala, Gly, DNP-Dap, DNP-Glu
13 → 18	Glu, Ala	+15 → +22	Dap, Glu, Ala, DNP-Dap, DNP-Ala
20 → 27	Glu, Ala, DNP-Dap, DNP-Ala	+24 → +26	Glu, Ala
30 → 42	Glu, Ala, DNP-Dap, DNP-Ala		

Dap = diaminopimelic acid. DNP = dinitrophenyl group. Glu = glutamic acid.
Ala = alanine. Gly = glycine.

Certain conclusions can be drawn from the results. Not every peptide fragment in the digests contained all the amino acids of the whole walls. This is contrary to results so far reported about the products of action of various bacteriolytic enzymes, where the non-dialysable peptides, produced by enzymic digestion of fresh walls or those already treated with fluoro-dinitrobenzene have been reported to contain all the constituents of wall mucopeptides (Salton, 1956*b*; Ingram & Salton, 1957; Koch & Dreyer, 1958). Digestion products from the action of λ -endolysin or lysozyme on dinitrophenylated walls of *Escherichia coli* and *Bacillus megaterium* were different from those produced from untreated walls (Work & Lecadet, 1960); the former therefore cannot be regarded as normal reaction products. The marked differences between the reaction products produced by λ -endolysin and lysozyme

Table 3. *Amino acid contents of some peptides in soluble fractions of λ -endolysin (from Escherichia coli K₁₂(λ) lysogenic mutant P₃₂) or lysozyme digests of walls of E. coli B.*

Samples (40 mg.) of walls were exhaustively digested with two successive portions of either enzyme in the presence of chloroform. After removal of insoluble products by centrifugation, digests were treated as in Table 2. The spots in the two digests were not identical, and are listed separately. The figures in the columns represent the distances (cm.) travelled by each spot.

λ -Endolysin products, dialysable		Lysozyme products, dialysable	
cm.	Amino acids	cm.	Amino acids
0	Dap, Lys, Asp, Glu, Ser, Gly		
4-5.5	Dap, Glu, Gly	3-5	Dap, Lys, Asp, Glu, Ser, Gly
6-7	Glu	6.5-7.5	Lys, Asp, Glu, Ser, Gly
8-10	Dap, Glu, Ser, Gly, Ala,* DNP-Dap	9-11	Lys, Asp, Glu, Ser, Gly, Thr, Ala, Val, Leu, Arg, Cys
16-18	Lys, Asp, Glu, Ser, Gly, Val, DNP-Lys, DNP-Ala	.	.
21-22	Glu, DNP-Dap	20-25	Lys, Asp, Glu, Ser, Gly, Thr, Ala, Val, Leu
30-36	Lys, Asp, Glu, Ser, Gly, Ala, Val, Leu, Arg, DNP-Dap, DNP-Lys, DNP-Glu, DNP-Ser, DNP-Ala	30-36	Lys, Asp, Glu, Ser, Gly, Ala, Val, Leu, Arg, DNP-Dap, DNP-Lys, DNP-Glu, DNP-Ser, DNP-Ala
36-42 (solvent front)	Lys, Asp, Glu, Gly, Thr, Ala, Val, Leu, DNP-Dap, DNP-Lys, Di-DNP-Lys, DNP-Glu, DNP-Ser, DNP-Ala	36-42 (solvent front)	Asp, Glu, Ser, Gly, Ala, DNP-Dap, Di-DNP-Lys, DNP-Glu, DNP-Ser, DNP-Ala

* Very large amount

non-dialysable		non-dialysable	
-2 \rightarrow +2	Lys, Asp, Glu, Ser, Gly, DNP-Dap	-2 \rightarrow +2	Lys, Asp, Glu, Ser, Gly, DNP-Dap
+ 8 \rightarrow +13	Dap, Asp, Glu, Ser, Gly, Ala, DNP-Dap	+ 8 \rightarrow +13	Dap, Glu, DNP-Dap,
+17 \rightarrow +21	Dap, Lys, Glu, Ser, Gly, Ala, DNP-Dap, Di-DNP-Dap, Di-DNP-Lys, DNP-Glu, DNP-Ser	+17 \rightarrow +21	Dap, Lys, Glu, Ala, DNP-Dap, Di-DNP-Lys, DNP-Glu, DNP-Ser
+21 \rightarrow +26	Dap, Glu, Ser, Gly, Ala, DNP-Dap, Di-DNP-Dap, DNP-Lys, Di-DNP-Lys, DNP-Glu, DNP-Ser, DNP-Ala	+21 \rightarrow +26	Dap, Lys, Glu, Ser, Ala, DNP-Dap, Di-DNP-Dap, DNP-Glu, DNP-Ser, DNP-Ala
+26 \rightarrow +28	Lys, Asp, Glu, Ser, Gly, DNP-Dap	+26 \rightarrow +28	Lys, Glu, Ser, Gly, DNP-Dap

Ala = alanine. Arg = arginine. Asp = aspartic acid. Cys = cystine. Dap = diaminopimelic acid. Glu = glutamic acid. Gly = glycine. Leu = leucine. Lys = lysine. Ser = serine. Thr = threonine. Val = valine. DNP = dinitrophenyl group.

from *E. coli* walls show that the enzymes are not identical in their action on this material, although they appeared to act identically on walls from *B. megaterium*. The reason for this is not known; it may be connected with the fact that walls of *E. coli* are the natural substrate for λ -endolysin. The lytic enzyme from T₂ coli phage was reported by Koch & Dreyer (1958) to resemble lysozyme in the fragments

which were produced from dinitrophenylated walls of *E. coli* B. Also, walls of *E. coli* already digested by T₂ coli phage enzyme were not further attacked by lysozyme, and *vice versa*. With λ -endolysin, on the contrary, the insoluble residues of dinitrophenylated walls of *E. coli* were further digested by lysozyme, while the lysozyme-resistant residues were also attacked by λ -endolysin. It is thus apparent that the lytic enzyme of λ -phage is different from that of T₂-phage. The results also indicated that *E. coli* walls are more complicated than was originally suggested by Weidel & Primosigh (1958). Each of the so-called 'layers' contained mucopeptide components which are partially, but not entirely, solubilized by lysozyme or λ -endolysin; lipids were also solubilized and were even found in dialysable fractions. Apparently many structures are held together in these walls by non-covalent links which are broken as a secondary result of solubilization of the mucopeptides. Under the influence of lytic enzymes the walls disintegrate into many types of fragments which differ according to whether lysozyme or λ -endolysin is acting.

The function of cell walls

When whole susceptible organisms are treated with lysozyme or other bacteriolytic enzymes the rigid mucopeptide component of the cell wall is attacked, and the immediate cause of lysis is the bursting of the mechanically unprotected cytoplasmic membrane by the internal osmotic pressure. When the digestion is carried out in isotonic solution, e.g. 10 % (w/v) sucrose, the cytoplasmic membrane remains intact and spherical forms are produced (see review by Weibull, 1958). With Gram-positive organisms the resulting bodies are known as protoplasts; chemical and immunological analyses have shown them to be free from wall components, including specific proteins and mucopeptides (Freimer, Krause & McCarty, 1959; Vennes & Gerhardt, 1959). Similar spherical bodies are produced from Gram-negative bacteria after appropriate preliminary sensitizing treatment, but cannot strictly be called protoplasts: not only do they still retain the wall lipoproteins and lipopolysaccharides, but they also contain mucopeptide components such as diaminopimelic acid and muramic acid (Salton, 1958); their membranes also react positively with cell-wall antibodies (Holme, Malmberg & Cota-Robles, 1960). The term spheroplast is now usually applied to the spherical osmotically sensitive forms from Gram-negative bacteria (Brenner *et al.* 1958). Protoplasts and spheroplasts are reasonably stable in media of sufficiently high osmotic pressure; they burst when transferred to water, leaving a residue of membranous ghosts, similar in size to the original body. The composition of the ghosts (cytoplasmic membranes) from Gram-positive bacteria is quite different from that of cell walls; they contain considerable quantities of lipids, but no hexosamines, wall sugars or diaminopimelic acid (Gilby, Few & McQuillen, 1958; Weibull & Bergstrom, 1958). The permeability properties of protoplasts or spheroplasts are the same as those of the bacteria from which they were derived, showing that their membranes are the osmotic barrier of the intact cell. Protoplasts under appropriate conditions can synthesize protein, can grow, divide and produce bacteriophage after infection or induction (McQuillen, 1955; Borek & Ryan, 1959). Thus they contain the main essential biosynthetic mechanisms of the cell, for which the wall mucopeptides are apparently not required when protection from osmotic effects is provided. Cell walls are permeable to dextrans of molecular weight up to 100,000 and to proteins excreted by the organisms,

suggesting that they have a sieve- or sponge-like structure. Their main function would appear to be that of a rigid porous envelope which protects the delicate cytoplasmic membrane within. Gerhardt (1959) and Butler, Crathorn & Hunter (1958) obtained evidence which suggests that certain amino acids may be stored in the walls; so walls may have the further function of a reservoir for certain metabolites.

Since the cell walls of all bacteria contain this specific mucopeptide structure, one ought to be able to prevent wall synthesis by growing exacting organisms in the absence of one of the mucopeptide components necessary for growth. This has been done with diaminopimelic acid (Meadow *et al.* 1957; Rhuland, 1957), lysine (Toennies & Shockman, 1958) and glucosamine (Zilliken, 1959). Meadow *et al.* (1957) used the two mutants of *Escherichia coli* (173-25 and D, see p. 171) which require to be given diaminopimelic acid for growth. When grown in glucose salts media supplemented with a constant (optimal) amount of lysine, and different amounts of diaminopimelic acid, cultures of mutant 173-25 had initial growth rates which were independent of the diaminopimelic acid concentration. Organisms grown in media with low concentrations of diaminopimelic acid lysed when the diaminopimelic acid was exhausted from the medium. Mutant D, grown in a similar series of media but without lysine, did not lyse, but just stopped growing when the diaminopimelic acid was used up. When lysine was present, lysis occurred on cessation of growth as with the cultures of mutant 173-25. This lysis can be explained on the basis that, provided some diaminopimelic acid is present, the organisms grow normally and form walls; as soon as the diaminopimelic acid is exhausted, new walls cannot be formed and the organisms burst, probably at the next division. The reason for the obligatory presence of lysine for lysis to occur is not known: it is not due to the fact that lysine allows cytoplasmic protein synthesis to continue so that the cell contents outgrow their walls (suggested by McQuillen, 1958*a*), since lysine can be replaced by α -N-acetyllysine as a growth factor, and in this case lysis does not occur.

When these diaminopimelic-requiring mutants are grown in the presence of sucrose and low concentrations of diaminopimelic acid, spheroplasts are formed (Meadow *et al.* 1957; Bauman & Davis, 1957; McQuillen, 1958*b*). These spheroplasts are able to grow, and to synthesize inducible enzymes in the presence of all amino acids except diaminopimelic, showing that this latter amino acid is not essential for protein synthesis.

Lysis through lysine deprivation occurs with *Streptococcus faecalis*, which contains no diaminopimelic acid, but has lysine instead in its wall mucopeptides (Toennies & Gallant, 1949; Toennies & Shockman, 1958). This organism requires to be given eight amino acids, including lysine, for normal growth; the effect of deprivation of each of these amino acids in turn was investigated. With suboptimal concentrations of lysine, the growth curves were very similar to those of the exacting *Escherichia coli* deprived of diaminopimelic acid, and the organisms lysed after exhaustion of the lysine present. A different phenomenon occurred with organisms depleted of valine or threonine, which are not wall amino acids (Shockman, Kolb & Toennies, 1958; Toennies *et al.* 1959; Shockman, 1959*a*). Exponential growth ceased when valine or threonine was exhausted from the medium, but the cultures continued to increase slowly in optical density and mass up to 40 hr. This so-called 'post-exponential growth' was particularly marked in the case of threonine-depleted organisms and was largely due to increase in wall mucopeptide with no concomitant

protein synthesis. Comparison of the molar ratios of the amino acids in the walls at various stages of growth indicated that the mucopeptides had the same composition at all stages. But when the amino acids were expressed as percentage of wall weight, it was evident that the overall composition of the wall had changed during post-exponential growth, possibly owing to variation of a non-mucopeptide component.

There is a tendency among workers in the field under consideration to examine only the molar ratios of specific wall constituents, not their absolute amounts in terms of percentage composition. This can be misleading, as it does not give a true picture of wall composition. More work of the kind carried out by Shockman and colleagues might lead to a better understanding of various interesting problems connected with cell walls, such as why bacteria harvested during exponential growth are more susceptible than older ones to attack by lytic enzymes (see Douglas & Parker, 1958). We do not know whether this is simply due to the presence of thicker walls in older organisms, or whether the older walls contain proportionately more enzyme-resistant structures or less enzyme-sensitive groups.

The biosynthesis of mucopeptide

The experiments just discussed show that synthesis of cell wall and cytoplasmic protein can continue independently in nutritionally-exacting organisms grown under certain conditions; this type of phenomenon has been termed 'unbalanced growth' (McQuillen, 1958*a*). Another way of dissociating the two types of synthesis is to suspend washed organisms in buffered glucose containing certain amino acids. This approach was used with *Staphylococcus aureus* by Mandelstam & Rogers (1958) and Hancock & Park (1958). Study of the incorporation of radioactive amino acids into walls and proteins of suspensions of *S. aureus* showed that when the amino acids of the suspending media were limited to those of the wall mucopeptides, radioactivity was incorporated selectively into the walls; but when 18 amino acids present in proteins were given, both walls and cytoplasmic proteins took up radioactivity. Added chloramphenicol inhibited 94 % of the uptake of amino acids into protein but had no effect on uptake into walls. Penicillin had the opposite effect and selectively inhibited the uptake of amino acids into the wall fraction. Aureomycin was quite unselective; it inhibited both wall and protein synthesis. Mandelstam & Rogers (1959) showed that the presence of penicillin or bacitracin prevented the usual increase in weight of mucopeptide observed during incubation, and therefore resulted in a genuine inhibition of mucopeptide synthesis.

As might be expected, since penicillin selectively inhibits the synthesis of a component of cell walls, the final result of penicillin action on bacteria growing in normal media is lysis. In fact, the growth curve of *Escherichia coli* in the presence of penicillin is similar to that produced by diaminopimelic acid deprivation (Meadow *et al.* 1957). Penicillin lysis has long been known, but the cause of it was not realized until spheroplasts of *E. coli* were produced by growth in penicillin broth containing 10 % sucrose (Lederberg, 1956; Liebermeister & Kellenberger, 1956; Hahn & Ciak, 1957). Bizarre forms of various Gram-negative bacteria growing on solid media in the presence of penicillin are well known; these may be regarded as spheroplasts which are stabilised mechanically by the solid medium in which they grow (Lederberg & St Clair, 1958). Analytical data on L-forms or penicillin-induced

spheroplasts show that the cells are deficient, but not always completely lacking in diaminopimelic acid and muramic acid (Kandler & Zehender, 1957; Weibull, 1958; Salton & Shafa, 1958). Their lipid and polysaccharide components are normal.

Before the site of penicillin action was known Park (1952) observed that when *Staphylococcus aureus* was grown in the presence of penicillin, certain uridine nucleotides accumulated in the cells. The main nucleotide was uridine diphosphate (UDP) linked through *N*-acetylmuramic acid to a peptide containing lysine, glutamic acid and alanine. At that time, the connexion of this structure with cell walls was not appreciated, nor was muramic acid identified. After the work of Strange & Powell (1954), Cummins & Harris, (1956*a, b*, 1958) and Lederberg (1956), a connexion with wall composition was seen (Park & Strominger, 1957); the nucleotide contained all the components of staphylococcal walls except glucosamine and glycine. The composition of the peptide has now been partly deduced (Fig. 1), although there is as yet no formal proof of the amino acid sequence (Strominger, 1959*a, b*; Strominger & Threnn, 1959).

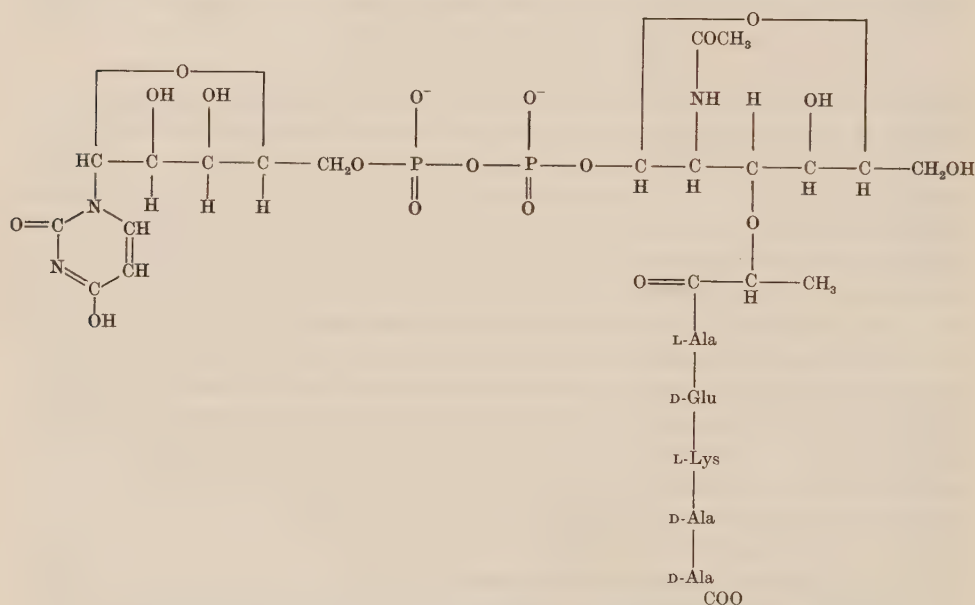


Fig. 1. The structure of a uridine nucleotide which accumulates in penicillin-inhibited *Staphylococcus aureus*.

The accumulation of other uridine nucleotides in *Staphylococcus aureus* grown under various conditions has led Strominger to propose a scheme for the biosynthesis of mucopeptide units (summarized by Strominger, 1960). This involves a 'UDP cycle' (Fig. 2) in which uridine nucleotides serve as catalytic carriers. For the purposes of this review, UDP-acetylmuramic acid can be taken as the starting point. This compound was assumed to originate from UDP-acetylglucosamine through UDP-acetylglucosamine-pyruvate (Strominger, 1958). UDP-acetylmuramic acid was one of several nucleotides accumulating in cells grown in the presence of gentian violet; it was suggested that gentian violet acts by inhibiting the attachment of alanine to the muramyl carboxyl group. After depriving *S. aureus* of lysine,

UDP-acetylmuramyl-L-alanyl-D-glutamate was isolated. An enzyme which catalyses the attachment of lysine to this peptide was partially purified, and found to be specific for L-lysine. D-Cycloserine (oxamycin), another antibiotic known to produce spheroplasts from *Escherichia coli* (Ciak & Hahn, 1959), caused accumulation of the uridine peptide which lacked only the two terminal D-alanine molecules (Strominger, Threnn & Scott, 1959). All the effects of D-cycloserine in different species were competitively reversed by adding D-alanine (see also Shockman, 1959*b*), but they were not produced by the enantiomorph, L-cycloserine. It seems that this antibiotic acts directly as an analogue of D-alanine. Finally, the last suggested step in the synthesis of wall mucopeptide is a transglycosylation of the UDP-acetylmuramyl-peptide with an acceptor, so far unidentified. It is this last step which is supposed by Park & Strominger to be inhibited by penicillin, but there is as yet no positive evidence for this assumption.

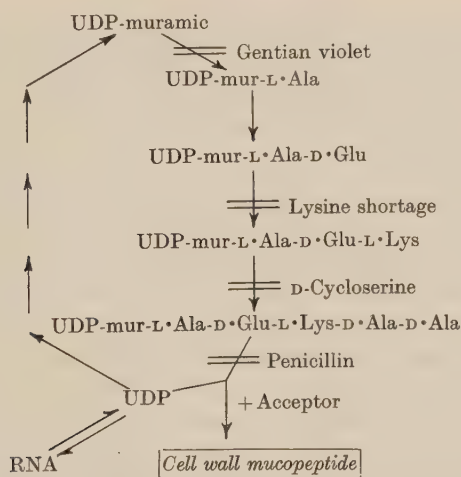


Fig. 2. Scheme for the biosynthesis of part of the cell wall of *Staphylococcus aureus*.

Other possible sites for penicillin action are inhibition of synthesis of the acceptor, or of transfer of one or more low-molecular weight precursors through the semi-permeable cytoplasmic membrane to the site of wall synthesis outside the membrane. Although UDP-peptide accumulation occurs very rapidly after addition of penicillin to growing or resting cultures (Strominger, 1957), this is not the only immediate effect of penicillin. There is also a very rapid release of ultraviolet-absorbing material into the culture fluid with *Escherichia coli*, which suggests that the osmotic barrier has been disturbed (Prestidge & Pardee, 1957; Meadow, 1960). Penicillin also interferes with the synthesis of ribonucleic acids in a way quantitatively related to total uridine nucleotide synthesis. This can be explained by the UDP-cycle, since the product of transglycosylation of the UDP-muramyl-peptide would be UDP, a direct precursor of ribonucleic acid. In the presence of penicillin, UDP would be irreversibly trapped in the accumulated uridine peptides and so would be unavailable for synthesis of ribonucleic acids; the inhibition of this synthesis is therefore a secondary result of penicillin action.

A transglycosylation step in mucopeptide synthesis is certainly an attractive

hypothesis; it is based on the knowledge that similar steps occur in the biosynthesis of low and high molecular-weight polysaccharides, including hyaluronic acid, glucan, cellulose and glycogen; here 'active' sugar fragments are transferred from uridine nucleotides to acceptors (Strominger, 1960). It is possible that this uridine peptide is indeed a precursor of wall mucopeptides, especially because of its content of D-amino acids, but it is not the only UDP-peptide found in *Staphylococcus aureus* treated with penicillin; similar peptides also contain the other staphylococcal wall amino acids, glycine and aspartic acid (Ito, Ishimoto & Saito, 1959). Direct evidence for the incorporation of any of these peptides as complete units into cell walls has yet to be produced as proof that the uridine peptides are, in fact, immediate cell-wall precursors. The method of insertion of glucosamine into the muramyl peptides is not yet known, but is an important step still to be revealed in mucopeptide biosynthesis.

Bacterial wall structure

Until we know more about the homogeneity of individual units of wall mucopeptides, it is perhaps not very useful to speculate about their possible precursors. The molar ratios of wall constituents are seldom exactly one, and their digestion products are of great variety (Rogers & Perkins, 1959; Perkins, 1960*b*). This suggests that whole walls may contain either a mixture of different highly polymerized mucopeptides, or one polymer made from various UDP peptides (monomers), or both. There may well be some favoured monomers which occur in many different organisms; these may be the 'basal units' of walls where there are also various other peptides derived from monomers which are more type-specific. The only uridine peptides which have been isolated from Gram-negative species have been found in a diaminopimelic acid-requiring mutant of *Escherichia coli* grown under unspecified conditions (Strominger, Scott & Threnn, 1959). One peptide had the same amino acid sequence as in the nucleotide peptide of penicillin-grown *Staphylococcus aureus* (Fig. 1) except that *meso*-diaminopimelic acid took the place of lysine. Under conditions of diaminopimelic deprivation, the peptide contained only L-alanyl-D-glutamic acid. Possibly the sequence acetylmuramyl-L-alanine-D-glutamic—(diaminopimelic or lysine)—D-alanine—D-alanine may be a favoured monomer which provides the 'basal unit' of walls of both Gram-positive and Gram-negative bacteria.

Perhaps a part at least of the mucopeptide structure can be envisaged as a backbone of *N*-acetylglucosamine—*N*-acetylmuramate, possibly in alternating (1 → 4), (1 → 6) β -glycosidic linkages, bound by peptide links through the carboxyl groups of muramic acid to various amino acids. This peptide link is often, but not invariably, through alanine: exceptions are the muramyl-glycyl sequence identified by Perkins & Rogers (1959) in partial acid-hydrolysates of walls of *Micrococcus lysodeikticus*, and the alanine-free peptides which were found in enzymic digests of *Escherichia coli* walls by Work & Lecadet (1960). The sugars of walls are also linked to the hexosamines and often are present as high molecular-weight lipopolysaccharides which are responsible for the group-specific antigenic properties of cell walls. Little is known about the sequences or compositions of the peptide chains except in the case of the uridine-peptides already described. There are few free amino groups in Gram-positive walls, usually that of alanine and one amino group of lysine or diaminopimelic acid (Ingram & Salton, 1957). Many of the pairs of amino groups of

lysine and diaminopimelic acid of *E. coli* are not free to react with fluorodinitrobenzene (Salton, 1957*b*; Work & Lecadet, 1960). This suggests that these amino groups act as cross-linking agents between adjacent chains, or form cyclic peptides. The cross-linking of chains is known to be one of the best ways of producing rigid structures, and this may be the function of the two amino acids lysine and diaminopimelic acid, which are the only long-chain amino acids in the mucopeptide structure. Lysine and diaminopimelic acid have identical chains—the latter compound having just one more carboxyl group; they appear to be mutually exclusive in the walls of Gram-positive organisms. Glutamic acid is invariably present in mucopeptides, but whether in α - or γ -linkages is not known. As a whole, the amino acids of mucopeptides present plenty of opportunity for cross-linking or cyclization; if not doubly linked they could tend to form strong non-covalent links either with adjacent mucopeptides or with other charged molecules such as the teichoic acids.

Table 4. *Amino acid components of walls of certain species of bacteria*

Glutamic acid and alanine were always present and are not included.

Genus	No. of species examined	Lysine	Diaminopimelic acid		Glycine
			Meso	LL	
<i>Staphylococcus</i>	9	+	—	—	+
<i>Micrococcus</i>	2*	—	+	—	+
<i>Propionibacterium</i>	4	—	—	+	+
<i>Propionibacterium</i>	1	—	+	—	—
<i>Bacillus</i>	3	—	+	—	—
<i>Clostridium</i>	1	—	—	+	+
<i>Nocardia</i>	4	—	+	—	—
<i>Nocardia</i>	1	—	—	+	+
<i>Micromonospora</i>	5	—	+	+	+

* Unstable species, diaminopimelic acid not always present.

+ = present; — = absent.

The D-configuration of the glutamic acid and alanine in walls is of great interest; it may account both for the stability of the mucopeptides against attack by proteolytic enzymes, and for the obviously different mechanisms of peptide biosynthesis in walls and in cytoplasmic proteins. The most common form of diaminopimelic acid is the *meso*-isomer with a D-configuration at one end and an L the other. In the *Escherichia coli* uridine-peptide, *meso*-diaminopimelic acid occurs between D-alanine and D-glutamic acid and might be linked in peptide bonds through its D 'end'. The unreacted L-end of diaminopimelic acid might either cross-link with L-amino acids in other peptides, or might retain the biological reactivity of the peptide for further biosynthesis by its capacity to react with enzymes in the usual L-configuration. This biological reactivity occurs in the decarboxylation and racemization of *meso*-diaminopimelic acid (p. 170), where the L 'end' of the molecule is thought to react with the enzymes. An interesting point arising from a study of the data about cell-wall composition is that where walls contain LL-diaminopimelic acid, glycine is invariably present, but when *meso*-diaminopimelic occurs, glycine is seldom found (Table 4). Walls of the species which contain LL-diaminopimelic acid have not yet been examined in detail, but if one assumes that they contain the D-isomers of

glutamic acid and alanine, it may be suggested that glycine, with its freely rotating amino and carboxyl groups might be able to align LL-diaminopimelic acid in a peptide chain containing D-amino acids.

CONCLUSION

In dealing with a structure—the cell wall—found in so many different types of organism, it is obvious that the work of a few years described here cannot give more than a preliminary sketch of the subject. This review, is therefore, not an attempt to cover the complete field, but deals only with certain topics in the hope that it may serve as a pointer for future work. One obvious need is for detailed analyses of mucopeptide components from a wide variety of bacterial species. Without this, no valid generalizations can be made.

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The Action of Acriflavine on Brewers' Yeasts

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SUMMARY

The absorption of acriflavine by strains of *Saccharomyces carlsbergensis* and *S. cerevisiae* occurred at approximately the same rate and extent and was greatest in the range pH 4–5. In this range, the proportion of respiration-deficient cells produced by acriflavine treatment was also maximal. Furthermore, under more alkaline conditions, the acriflavine exerted a pronounced toxic effect. At pH 4–5, the proportion of mutant cells produced was about 50 times greater in strains of *S. cerevisiae* than in those of *S. carlsbergensis*, while fewer cells were killed. With *S. cerevisiae* the production of mutants was rapid and in certain strains exposure for 1 hr. to 50 µg. acriflavine/ml. led to about one-third of the cells mutating, while after 4 hr., all the cells were respiration-deficient. In contrast, exposure of strains of *S. carlsbergensis* for 24 hr. at this concentration of acriflavine induced only about one-tenth of the cells to mutate. The mutants took up no measurable amount of oxygen when examined in Warburg respirometers. As compared with the parent cultures, there was no difference in the ability of the cells to utilize carbohydrates and to undergo flocculation.

INTRODUCTION

Ephrussi and co-workers studied respiration-deficient mutants of bakers' yeast which they called 'la mutation petite colonie' (summary, Ephrussi & Hottinguer, 1951), while Lindegren and his associates refined the techniques for detecting such mutants (Ogur, St John & Nagai, 1957). Furthermore, the latter, by the use of selective media, demonstrated the effectiveness of a number of agencies in inducing this mutation (Ogur, Lindegren & Lindegren, 1954; Ogur & St John, 1956; Nagai & Nagai, 1958). With this background, the present work was concerned with the mode of action of acriflavine (euflavine), probably the most potent of the relevant agents. From a study of the absorption of acriflavine, its toxicity, and its ability to induce respiration-deficiency in a range of brewing yeasts, quantitative differences in its effect on *Saccharomyces cerevisiae* and *S. carlsbergensis* were observed. An attempt has been made to define the optimal conditions for such effects.

METHODS

Organisms. The following yeasts, obtained from the British National Collection of Yeast Cultures (NCYC), were used:

Saccharomyces cerevisiae NCYC, nos. 239, 1062, 1063, 1108 and 1109.

Saccharomyces carlsbergensis NCYC, nos. 396, 397, 398, 399 and 400.

These strains were selected to cover a range of behaviour, certain aspects of which are important under brewery fermentation conditions, namely ability to form flocs, yeast heads, and chains of cells (Eddy, 1958).

Media. The yeasts were maintained as stock cultures in a liquid malt extract + yeast extract + glucose + peptone medium (MYGP; Wickerham, 1951). Before testing, active cultures were prepared by transferring a small inoculum (0.25 ml.) to fresh medium (100 ml.) and incubating at 25° with gentle shaking for 18 hr. Investigations were mostly conducted in a defined medium (A) with a normal pH of 4.5 described by Kilkenny & Hinshelwood (1951). Where precise conditions of pH stability were required equal volumes of M/15 citrate + phosphate buffer and double strength medium A were mixed.

Stock solutions of acriflavine (B.P.C. quality) in distilled water at concentrations of 100 and 1000 $\mu\text{g./ml.}$ were kept in the dark. They were sterilized by steaming on three successive days, and the concentrations adjusted by measurement of optical density at 4400 Å., followed by appropriate dilution with sterile distilled water.

Detection of respiration-deficient colonies. After suitable treatment, cell populations were diluted and plated on MYGP agar, and incubated for 48 hr. at 25°. The colonies were then overlaid with a solution of triphenyl tetrazolium chloride (TTC) solidified with agar, and buffered to pH 7 (Ogur *et al.*, 1957). Glucose (0.5%, w/v) was added to the overlay mixture for improved resolution of mutant (white) and normal (red) colonies (Nagai, 1959).

Estimation of viability and growth rate in solid media. Suspensions of yeast in media containing gelatin were prepared and examined microscopically over a period of 4–5 hr. at 18° in a haemocytometer (Gilliland, 1959). Suspensions were prepared in the appropriate medium, with or without acriflavine, containing 6% (w/v) gelatin and held at 25° to preserve the liquid state. While still liquid, one drop of a suspension was transferred to a haemocytometer and the edges of the cover-glass sealed with vaseline. After a period to allow the gelatin to set, the preparation was examined microscopically and the position of each cell noted.

Absorption of acriflavine. Suspensions of yeast cells ($10^8/\text{ml.}$) were prepared, in M/15 citrate + phosphate buffer of appropriate pH, together with glucose (2%, w/v) when required. The suspensions were kept agitated with a wrist action shaker. At zero time a solution of acriflavine was added to 50 $\mu\text{g./ml.}$ Samples were removed at once, and at suitable intervals subsequently, and centrifuged. A measured sample of the clear supernatant liquid was diluted appropriately and the optical density (OD) determined, to provide a measure of acriflavine concentration. At 4400 Å. there was a linear relationship between OD and acriflavine concentration within the range 0–10 $\mu\text{g./ml.}$

Measurement of carbon dioxide evolution and oxygen consumption. These were carried out in a conventional Warburg apparatus, at 25°. Yeasts were grown in MYGP medium for 18 hr. at 25° in gently shaken culture, centrifuged, and re-suspended in M/15 KH_2PO_4 solution (pH 4.5). Cell material corresponding to 2 mg. dry wt. was used in each vessel. Respiratory substrates (2%, w/v) were prepared in a similar KH_2PO_4 solution.

Growth rate of respiration-deficient strains in liquid media. This was followed by turbidimetric estimation of suitably diluted samples.

RESULTS

Production of respiration-deficient strains of Saccharomyces cerevisiae and S. carlsbergensis

Strains of the yeasts were exposed to acriflavine at concentrations of 10, 50 and 100 $\mu\text{g./ml.}$ in medium A at pH 4.6 for 4 hr. A cell concentration of $10^6/\text{ml.}$ was used. The typical results shown in Table 1 demonstrate that acriflavine had a pronounced mutagenic effect on the strains of *Saccharomyces cerevisiae* used, but only a small one on *S. carlsbergensis*. These studies were repeated on two strains of

Table 1. *Production of mutants of Saccharomyces cerevisiae and S. carlsbergensis by acriflavine*

Cultures were treated with different concentrations of acriflavine for 4 hr. at 25° C. After dilution and plating, the resultant colonies were overlaid with a mixture of agar and tetrazolium salt. 'Sectored' colonies are those derived from juxtaposed mutant and normal cells.

		Acriflavine ($\mu\text{g./ml.}$)					
		10		50		100	
		% mutant colonies	% sectored colonies	% mutant colonies	% sectored colonies	% mutant colonies	% sectored colonies
<i>S. cerevisiae</i>	239	9	22	86	12	100	0
	1026	0	9	98	2	—	—
	1108	90	9	77	16	94	6
<i>S. carlsbergensis</i>	396	1	25	0	28	1	5
	398	6	7	6	12	15	19
	399	0	0	0	0	0	0

S. carlsbergensis with the exposure time lengthened to 24 hr., but respiration-deficient cells still made up only a small proportion of the final population (Table 2). 'Sectored' colonies where they occurred could be accounted for by the tendency of certain strains to form either chains of cells, or flocs, resulting in the juxtaposition of unchanged and mutant cells. This inevitably makes it difficult to draw quantitative conclusions where sectoring was common; therefore, in the majority of trials, the strains *S. carlsbergensis* 396 and *S. cerevisiae* 239, with reasonably unclustered growth habits, were used.

Table 2. *Production of mutants of Saccharomyces carlsbergensis by acriflavine*

Experimental procedure was as in Table 1, except that the cells were exposed to acriflavine for 24 hr.

		Acriflavine ($\mu\text{g./ml.}$)					
		10		50		100	
		% mutant colonies	% sectored colonies	% mutant colonies	% sectored colonies	% mutant colonies	% sectored colonies
<i>S. carlsbergensis</i>	396	9	2	40	6	42	7
	399	0	29	1	96	—	—

Characteristics of respiration-deficient strains

Cultures obtained by transfer of 'white' colonies after TTC overlay were maintained on slopes of MYGP agar after two transfers in MYGP broth with checking by TTC overlay at each transfer. Their stability was demonstrated by regular testing during periods of up to one year using the TTC overlaying technique and manometric measurements of gas exchange. These tests revealed consistent inability either to reduce TTC or absorb oxygen.

Colony size could not be measured objectively, but diameters of the order of one-fifth that of the parent strains were regularly observed after a 48 hr. incubation period.

Spectroscopic examination of respiration-deficient strains, grown in MYGP under aerobic conditions at 25°, revealed the existence of a strong absorption band at 5500 Å., corresponding to reduced cytochrome *c*; bands at 6000 and 5620 Å. corresponding to the *a* and *b* components were not visible. All the bands were evident when the parent strain was examined.

It was therefore concluded that the cytochrome complement of the respiration-deficient cultures is similar to that of the 'petite colonic' strains described by Slonimski (see Ephrussi, 1953).

Further evidence of the similarity to the 'petite' strains was provided by the inability of the respiration-deficient strains to grow in media with acetate or lactate as carbon sources.

Demonstration of the direct mutagenic action of acriflavine

The presence of a high proportion of respiration-deficient cytoplasmic mutants in a cell population that had been exposed to acriflavine might be due to: (a) accelerated growth of pre-existing mutants; (b) direct mutagenic action; (c) accelerated growth of induced mutants. Nagai & Nagai (1958) suggested that the first alternative is unlikely and the following investigation shows that only the second alternative is applicable.

Saccharomyces cerevisiae 239 and *S. carlsbergensis* 396 and respiration-deficient mutants derived from them, 239A and 396A, were examined by means of the slide-culture technique after it had been established that substantially all the cells were viable. The relative number of dead cells was taken as the proportion of cells staining after suspension in 0.01 % (w/v) methylene blue, buffered at pH 5.0. The increase in numbers of normal and mutant cells with and without acriflavine present was followed for a period equivalent to that used in the preparation of mutants (Table 3). Clearly, in the presence of acriflavine the respiration-deficient cells were inferior in their rate of cell division to the corresponding normal cells and, far from being selected, are placed at a severe disadvantage. Their presence must therefore have been a direct effect of the acriflavine. Further, it will be recalled that Table 1 shows, for example, 86 % of respiration-deficient cells in the entire population of *S. cerevisiae* 239 after treatment with acriflavine (50 µg./ml.) for 4 hr. Table 3 shows that in this period cell numbers have increased by 76 %. Hence an original population of 100 cells has increased to 176 cells and 86 % of these (151 cells) are respiration-deficient. It follows, therefore, that at least 51 of the original population had mutated.

Table 3. *Growth of yeasts whilst exposed to acriflavine*

Haemocytometer slides were set up containing yeast suspensions in gelatin-solidified medium A, together with appropriate amounts of acriflavine. The cultures were incubated at 18° for 4 hr., and examined microscopically at the beginning and end of this period. Buds having a diameter less than one-fifth of the parent cell were not counted.

Yeast strain	Acriflavine ($\mu\text{g./ml.}$)			
	0	10	50	100
	Increase in cell number as % original no.			
<i>Saccharomyces cerevisiae</i> 239	140	96	76	68
239 A	100	68	20	5
<i>S. carlsbergensis</i> 396	128	66	23	11
396 A	28	12	0	0

The optimal conditions for the production of respiration-deficient cytoplasmic mutants

Effect of pH on mutagenic action. *Saccharomyces cerevisiae* 239, and *S. carlsbergensis* 396 were incubated in medium A at a range of concentrations of acriflavine, and at pH values between 4 and 8. The incidence of respiration-deficient colonies in the suspension was estimated by the overlay technique (Tables 4 and 5). Clearly mutation was most pronounced at low pH values, but the effect of increasing alkalinity was not only to decrease mutagenic action but also to increase toxicity (Albert, 1951).

Table 4. *The effect of pH on the mutagenic action of acriflavine on Saccharomyces cerevisiae 239*

Tubes were set up containing yeast suspension in medium A buffered appropriately and acriflavine at 50 or 100 $\mu\text{g./ml.}$; incubation for 4 hr. at 25°. After incubation, the suspensions were diluted and plated on MYGP agar, incubated at 25° for 48 hr. and overlaid with TTC agar. The results given are the means of six replicates.

Acriflavine ($\mu\text{g./ml.}$)	pH value of medium					
	4		6		8	
	Colonies per plate	% mutants	Colonies per plate	% mutants	Colonies per plate	% mutants
0	465	0	357	0	262	0
50	353	84	151	32.5	96	22
100	262	97	131	42	68	54

The original suspension gave 188 colonies/plate, all normal.

Effect of pH value on the absorption of acriflavine. A study of absorption of acriflavine over a range from pH 3 to 8 revealed no quantitative or qualitative differences between strains *Saccharomyces cerevisiae* 239 and *S. carlsbergensis* 396; the results for the former strain are shown in Fig. 1. The optimal pH value for absorption, pH 5, was also similar in the two strains. This was close to the optimal pH value for mutagenic action. On the other hand, a considerable absorption took place at pH 8, when the acriflavine was more toxic and little mutation occurred.

Table 5. *The effect of pH on the mutagenic action of acriflavine on Saccharomyces carlsbergensis 396*For experimental details, see Table 4. Period of incubation, 24 hr.
pH of medium

Acriflavine ($\mu\text{g./ml.}$)	4		5		6		8	
	Total no. per plate	% mutants	Total no. per plate	% mutants	Total no. per plate	% mutants	Total no. per plate	% mutants
0	230	0	190	0	370	0	150	0
10	90	13	30	15	No growth	—	No growth	—
50	33	9	2	0	No growth	—	No growth	—
100	17	5	3	3	No growth	—	No growth	—

Glucose had no effect on the rate or extent of the absorption. The mutagenic action at pH value of 3 and less was not examined since little absorption took place.

Viability after exposure to acriflavine. Since *Saccharomyces cerevisiae* and *S. carlsbergensis* differed in their susceptibility to the mutagenic effect of acriflavine, it seemed possible that they might differ also in sensitivity to the toxic action of the mutagen. Estimates of the toxic effect were obtained by using the slide-culture technique. Yeasts were exposed to acriflavine whilst in medium A, and were washed three times in saline solution before microscopic examination. The results (Table 6) show that *S. cerevisiae* was resistant to the toxic effect of acriflavine whilst being susceptible to the mutagenic effect, the reverse applying with *S. carlsbergensis*.

Table 6. *Viability of yeast after exposure to acriflavine for 4 hr.*

Yeast strain	Acriflavine ($\mu\text{g./ml.}$)	Cells observed initially	Non-budding cells observed after 5 hr.	Viability %
<i>Saccharomyces cerevisiae</i> 239	0	82	3	96
	10	106	2	98
	50	89	10	89
	100	80	5	94
<i>S. carlsbergensis</i> 396	0	85	0	100
	10	110	10	91
	50	94	34	64
	100	103	44	58

The rate of production of respiration-deficient cells. To follow the pattern of appearance of mutants with time at different initial acriflavine concentrations, *Saccharomyces cerevisiae* 239 was used since it is particularly susceptible to the mutagenic effects over short periods during which the toxic effects are negligible (Fig. 2). The considerable incidence of mutants after 1 hr. at an acriflavine concentration of 50 $\mu\text{g./ml.}$ is of interest because less than 12% of the cells have cleaved during that period in the presence of acriflavine (Table 7). A second feature is the limited mutagenic effect of acriflavine at 10 $\mu\text{g./ml.}$; at the cell density used such an initial concentration never led to the whole population mutating.

On increasing the concentration of the yeast suspension at constant acriflavine concentration, the production of mutants was progressively decreased.

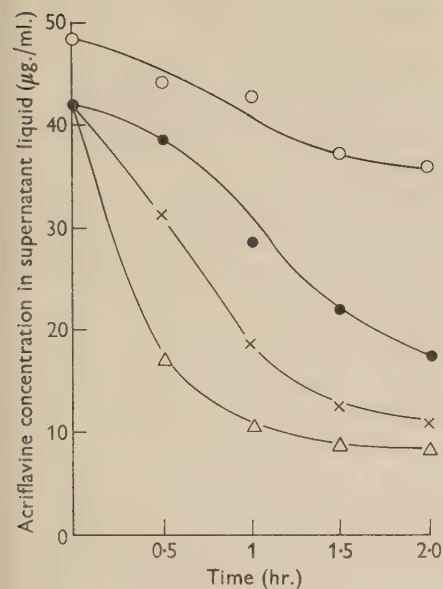


Fig. 1

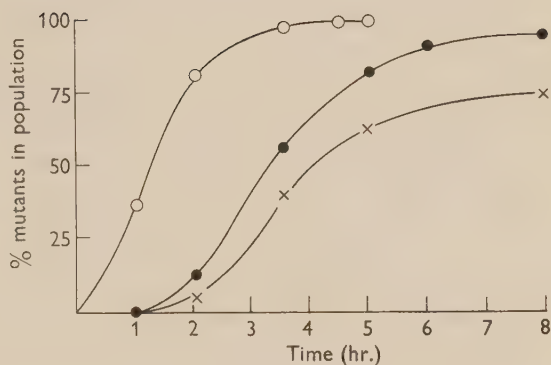


Fig. 2

Fig. 1. The absorption of acriflavine by *Saccharomyces cerevisiae* 239. The system comprised: 100 ml. yeast (10^8 cells/ml.) suspended in M/15 citrate + phosphate buffer. Acriflavine solution was added at zero time, and a sample removed for assay. Suspensions were shaken at 20° . ○ = pH 3; △ = pH 5.0; × = pH 7.0; ● = pH 8.0.

Fig. 2. The production of respiration-deficient mutants with time. The system comprised: *Saccharomyces cerevisiae* 239, suspended in medium A + acriflavine. Cell concn. 10^6 /ml. Total volume 10 ml. Samples (0.5 ml.) withdrawn at times shown. Suspensions shaken gently at 25° . Acriflavine ($\mu\text{g./ml.}$): ○ = 50; ● = 20; × = 10.

Selected physiological characteristics of mutant strains

Estimates of the endogenous gas exchange were made over 30 min. in the Warburg apparatus. The quotients $Q_{O_2}^{\text{air}}$, $Q_{CO_2}^{\text{air}}$, and $Q_{CO_2}^{N_2}$ were then measured over the period 10–40 min. after addition of glucose. No measurable endogenous gas exchange was observed with the ten strains tested. The $Q_{CO_2}^{\text{air}}$ and $Q_{CO_2}^{N_2}$ values for any one strain were the same, and for all strains were within the range 150–200 $\mu\text{l. CO}_2$ /mg. dry wt./hr. No oxygen uptake was ever observed. However, Slonimski (1958) quoted a diminution of oxygen consumption of 98%, and a residual uptake

Table 7. *Growth of yeast in liquid medium + acriflavine*

System comprised *Saccharomyces cerevisiae* 239 suspended in medium A + acriflavine (50 $\mu\text{g./ml.}$). Cell concn. 10^6 /ml. Suspension shaken gently at 25° . At intervals, samples were removed aseptically, diluted, and plated on MYGP agar. Colonies scored after incubation for 48 hr. at 25° . Figures are means of six replicates.

Time	Colonies
0	102
1	114
2	125
4	180

of 2% (about 4 μ l./vessel/hr.) would not be measured with certainty in a conventional Warburg respirometer.

Fermentation characteristics. Four mutant strains were grown on brewers' wort (containing principally fructose, sucrose, glucose, maltose, maltotriose and maltotetraose), and their ability to utilize these constituents compared with that of the equivalent normal strain. No differences were detected.

Flocculation. Eight strains of respiration-deficient mutants showed no change in this characteristic when compared with the equivalent parent strain.

DISCUSSION

Ephrussi & Hottinguer (1950) contended that when a population of yeast cells is exposed to acriflavine, respiration deficiency is only manifested in those daughter cells which are produced during the period of exposure or shortly afterwards. The parent cells appear not to be affected by the mutagen and retain their normal respiratory abilities. However, in the present work there is some evidence for direct mutagenic action on the parent cells. Thus from Fig. 2 it is evident that a high proportion of mutants appeared in a population of *Saccharomyces cerevisiae* which was exposed to acriflavine at 50 μ g./ml. for 1–2 hr. under conditions where little cell division had occurred (Table 7) and the viability was over 94% (Table 6).

Differences between *Saccharomyces carlsbergensis* and *S. cerevisiae* with regard to the mutagenic and toxic actions of acriflavine are not associated with the rates of adsorption of the dye. Perhaps the organisms differ instead in the rate at which acriflavine penetrates the cytoplasmic membranes. However, since in *S. carlsbergensis* relatively few mutants were produced after long exposure, this explanation is not entirely satisfactory. It would seem much more likely that there is a difference between the two species in the materials responsible for synthesis of, for example, cytochrome components. A further explanation can be suggested, namely that equal numbers of mutants are initially produced by the two species but that many of these newly formed mutants of *S. carlsbergensis* are incapable of division. It is, however, unlikely that direct evidence for this explanation can be obtained.

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The Function of Glycerol, Cholesterol and Long-Chain Fatty Acids in the Nutrition of *Mycoplasma mycoides*

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SUMMARY

Mycoplasma mycoides var. *mycoides* requires for growth a number of preformed lipid precursors. Media containing glycerol, cholesterol, a saturated and an unsaturated fatty acid, defatted bovine serum albumin and an additional defatted serum protein fraction (Fraction C) can supply these requirements. Albumin is believed to function by binding fatty acids, and Fraction C by binding cholesterol. The requirement for a saturated fatty acid can be satisfied by myristic, palmitic, stearic or margaric acid, lauric acid being less effective. The requirement for an unsaturated fatty acid can be satisfied by oleic acid, linoleic and linolenic acids being less effective. Organisms incubated in a medium deficient in either glycerol, the Fraction C+cholesterol system, or oleate, but adequate with respect to all other nutrients, died rapidly. Death was accompanied by lysis. Death due to a deficiency of glycerol or of cholesterol was prevented either by the omission of uracil (an essential nutrient) or by addition of chloramphenicol. Death due to oleate deficiency was not prevented by the omission of uracil. Morphological changes which resulted from each of these deficiencies are illustrated by electron micrographs. The hypothesis is advanced that glycerol, cholesterol and long-chain fatty acids are all needed for the synthesis of an undetermined cell component which is necessary for the structural integrity of the cell, and that the synthesis of this is more sensitive to a deficiency of these nutrients than is the synthesis of cytoplasm.

INTRODUCTION

A medium of partly defined composition which supported good growth of *Mycoplasma mycoides* was described by Rodwell (1960). It contains a heat-stable defatted serum protein fraction (Fraction C), cholesterol, an unsaturated fatty acid (or 'Tween 80'), serum albumin, glycerol, high concentrations of DL- or L-lactate and glucose. It enabled requirements for adenine, guanine, uracil, thymine, riboflavin, thiamine, nicotinic acid, α -lipoic acid, pantothenic acid and biotin to be recognized. The amino acid requirements were not defined. The function of the medium components which serve as lipid precursors has now been investigated.

The concentration of glycerol required for maximum growth increased from about

5 $\mu\text{g.}/\text{ml.}$ in static cultures to about 50 $\mu\text{g.}/\text{ml.}$ in cultures rotated to give a moderate degree of aeration during incubation (Rodwell, 1960). Glycerol is rapidly oxidized to acetate and CO_2 by suspensions of *Mycoplasma mycoides* grown aerobically. Evidence for a flavoprotein-catalysed oxidation of glycerophosphate was reported by Rodwell & Rodwell (1954). If this pathway were irreversible, glycerophosphate could not be formed from hexose, and an exogenous source of glycerol would be needed for lipid synthesis. Experiments have shown that (^{14}C)-labelled glycerol is incorporated, probably without dilution from hexose, into lipids by growing cultures of *M. mycoides* (Plackett, 1961). The quantitatively greater requirement for glycerol under aerobic growth conditions is due to the greater rate of oxidation and consequent loss of glycerophosphate for synthetic reactions. The observation that cultures of *M. mycoides* undergo rapid lysis when growing under conditions of glycerol deficiency, but not when some other nutrients are growth-limiting, suggested that glycerol deficiency might cause the phenomenon known as unbalanced growth.

The function of sterols in the growth of mycoplasma organisms is of considerable interest, since other bacteria are not known to require or to synthesize them, except possibly in trace amounts by some species (Dauchy, Kayser & Villoutreix, 1956; Fiertel & Klein, 1959). Smith (1959, 1960) reported both cholesterol esterase (ester-synthesizing) and lipase (ester-splitting) activity in a strain of human origin, and Lynn & Smith (1960) studied the distribution of esterified and free cholesterol between the soluble and insoluble fractions of suspensions of disrupted organisms from several strains. Their results indicated that both free and esterified cholesterol were present in both fractions. Sterol was not detected in two strains of *Mycoplasma laidlawii* which did not require sterol for growth. It was suggested previously that cholesterol exercised a protective function during the growth of *M. mycoides*. From the experimental data it could not be determined whether cholesterol is required as a nutrient in the strict sense (i.e. whether it is incorporated into the cellular structure) in addition to the postulated protective function (Rodwell, 1956). A detoxifying function for cholesterol was also suggested to explain its growth-promoting effect for *M. laidlawii* (Butler & Knight, 1960; Razin & Knight, 1960).

A requirement for an unsaturated fatty acid for the growth of *Mycoplasma mycoides* was also postulated previously (Rodwell, 1956). The partly defined medium (Rodwell, 1960) contains bovine serum albumin and 'Tween 80'. The fatty acid requirements and the functions of serum albumin and of fatty acids have now been investigated.

METHODS

Organism. The strain used was the V5 strain of *Mycoplasma mycoides*, isolated from a case of bovine pleuropneumonia in 1936.

Medium A. The composition of a partly defined medium was previously reported (Rodwell, 1960). Details for its preparation are now described. It contains: $\text{Na}_2\text{HPO}_4 + \text{KH}_2\text{PO}_4$ (pH 7.8), 0.04M; Na DL-lactate, 0.14M; cholesterol, 10 μM ; and (per l.) glucose, 5.0 g.; bovine serum Fraction C, 0.5 g.; bovine serum albumin Fraction V (Armour), 2.0 g.; Tween 80, 5.0 mg.; glycerol, 0.05 g.; acid-hydrolysed casein (Difco, vitamin-free), 2.5 g.; tryptic digest of casein, \equiv 2.0 g. casein; L-cystine, 2.5 mg.; L-tryptophan, 5.0 mg.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 50 mg.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25 mg.; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.25 mg.; adenine, 5.0 mg.; guanine, 5.0 mg.; uracil,

5.0 mg.; thymine, 2.5 mg.; Ca pantothenate, 0.25 mg.; riboflavin, 0.25 mg.; pyridoxal-HCl, 0.05 mg.; nicotinamide, 0.5 mg.; thiamine, 0.5 mg.; biotin, 0.01 mg.; DL- α -lipoic acid, 0.01 mg. The medium was distributed in 20 mm. diameter optically matched test tubes covered with aluminium caps and sterilized by autoclaving at 10 lb./sq.in. for 10 min. Bovine serum Fraction C was added to the medium before autoclaving. Bovine serum albumin Fraction V and glucose were added from sterile (filtered) solutions after autoclaving the bulk of the medium. The final volume after these additions was 5 ml./tube. The pH value of the medium was 7.5.

Medium B. As a result of the studies on the fatty acid requirements to be described medium A was modified. Bovine serum albumin Fraction V was replaced by Fraction V extracted by *iso*-octane + glacial acetic acid mixture (19 + 1; Goodman, 1957) at the decreased concentration of 1.0 g./l. Tween 80 was replaced by Na palmitate + Na oleate each at 20 μ mole/l. Inositol (1 mg./l.), choline chloride (1 mg./l.) and leucovorin (folinic acid) (0.25 mg./l.) were also added.

Preparation of medium constituents. Na DL-lactate was prepared from CP or AR grade lactic acid. The Zn salt was made, twice recrystallized, decomposed with sodium carbonate, and the remaining traces of zinc removed by successive treatments with 8-hydroxyquinoline in chloroform (Waring & Werkman, 1942). Excess carbonate was then decomposed by boiling with additions of HCl until the pH value remained at about 7.0. Alternatively, the Zn salt was suspended in water and shaken with a small excess of ion-exchange resin (Amberlite IR. 120, H⁺ form). The resin was drained, washed, the supernatant fluid and washings passed through a small column of fresh resin, and the acid titrated to pH 7.0 with NaOH. Solutions prepared by either method had equal activity. Several samples of CP and AR grade lactic acid when tested without purification inhibited growth at higher concentrations.

Bovine serum Fraction C was made from ox serum. Serum was diluted with an equal volume of water, heated to 70°, cooled, adjusted to pH 5.6 with 5% (v/v) acetic acid, heated to 80°, cooled, and filtered on a Büchner funnel. The filtrate was saturated with ammonium sulphate. The orange-coloured precipitate which rose to the surface was collected by gravity filtration through folded Whatman no. 2 paper. It was suspended in a small volume of water and the suspension dialysed thoroughly against four to six changes of distilled water. The dialysed solution containing some insoluble material was adjusted to pH 4.8–5.0, whereupon about half the protein was precipitated. The suspension was chilled to 0–2°, and 10 vol. cold ethanol added slowly. The precipitate was washed twice with the same volume of ethanol at –5° on the centrifuge, then successively with ethanol, ethanol + ether mixture, and ether on a Büchner funnel. The yield of cream-coloured powder was 1.5–2.0 g./l. serum taken. The product was stable indefinitely. For use in the medium, it was ground in a mortar with water, 0.05N-NaOH added to pH 7.4 (about 7.0 ml./g. powder), and the mixture stirred for about 30 min. The concentration was adjusted to 1% (w/v) with water, and a small amount of insoluble material centrifuged off and discarded.

Cholesterol was added to the medium as a colloidal dispersion in water. One volume of a 20 μ M solution in ethanol at 60° was injected rapidly into 19 vol. of stirred water at 60°. Cholesterol dispersions were stored in evacuated Thunberg tubes in a refrigerator. To avoid precipitation it was necessary to add the cholesterol dispersion to the medium after the bovine serum Fraction C had been added.

Tween 80 was purified as described by Davis (1947).

Oleic acid was purified from commercial redistilled oleic acid by precipitation of the long chain saturated fatty acids from acetone at -20° , followed by five recrystallizations of the unsaturated acid from acetone at -60° (Brown & Shinowara, 1937).

Linoleic and linolenic acids were prepared by saponification followed by bromination of the fatty acid mixtures, and would therefore be a mixture of the *cis* and *trans* isomers.

Lauric, myristic, palmitic and stearic acids were Eastman-Kodak products and were recrystallized several times from acetone. Margaric acid was a product of L. Light and Co. (Colnbrook, Bucks). Fatty acid impurities were not detected in any of the acids by reversed-phase circular paper chromatography as described by Nowotny, Lüderitz & Westphal (1958). Margaric acid migrated at a speed intermediate between those of palmitic and stearic acids in this solvent system. The fatty acids were added to the medium as the Na salts. Sparingly soluble salts were warmed to dissolve them before adding them to the medium.

Bovine serum albumin Fraction V (Armour) was heated at 56° for 30 min. at pH 7.0 to inactivate lipase (Davis & Dubos, 1946). Bound fatty acids were extracted with *iso*-octane containing glacial acetic acid as described by Goodman (1957). The fatty acid content of the albumin after extraction was not determined.

Tryptic digest of casein was made from 'vitamin-free' casein (Glaxo) and was charcoal-treated as described by Roberts & Snell (1946).

The other reagents were commercial products. Stock solutions were stored in a deep-freeze cabinet and were replaced at monthly intervals.

Preparation of inocula. Organisms for inocula were grown in BVF-OS medium (Turner, Campbell & Dick, 1935). Cultures were centrifuged at 15,000g for 15 min., the tubes drained, the deposit washed once with 0.4M-sucrose solution containing 0.01M-phosphate buffer (pH 7.0) and resuspended in sucrose + phosphate solution.

Growth tests. Duplicate tubes were seeded with about 2×10^6 viable elements/ml., and the growth estimated turbidimetrically at 660 m μ at intervals during static incubation at 37° . Streaming birefringence was judged visually by the amount of 'swirl' when the cultures were gently agitated. In many instances the cultures were examined by dark-field microscopy.

Nutritional deficiency experiments. Replicate tubes of partly defined medium with the additions or omissions indicated in the text were seeded with inocula of 5×10^7 to 5.5×10^8 viable elements/ml. and were incubated at 37° in an upright position without shaking. One tube of each replicate set was used for each viable count or for the preparation of specimens for electron microscopy. Two tubes of each replicate set, and also two tubes of uninoculated medium, were reserved for turbidity measurements at 440 m μ .

Counts of viable elements. These were made by a modification of the Miles & Misra (1938) method, as adapted for counting *Mycoplasma mycoides* by Mr G. S. Cottew (personal communication).

Preparation of specimens for electron microscopy. The cultures were chilled to 0° , centrifuged in the cold, the pellets resuspended in sucrose + phosphate solution and the suspensions squirted into 10 vol. of sucrose + phosphate containing 4% (w/v) formaldehyde. After standing for 4–18 hr. at room temperature they were centrifuged and the pellets washed twice and resuspended in distilled water.

RESULTS

Effect of glycerol deficiency

Tubes of (i) Medium A, (ii) Medium A lacking glycerol, (iii) Medium A lacking uracil, and (iv) Medium A lacking both glycerol and uracil, were seeded with 5.5×10^8 viable elements/ml. Viable element counts and turbidity measurements were made at intervals during incubation for 24 hr. During this period, the viable count increased about fourfold in (i), decreased to about 15% of the number inoculated in (ii), and remained almost stationary in (iii) and (iv) (Fig. 1*b*). That is, a

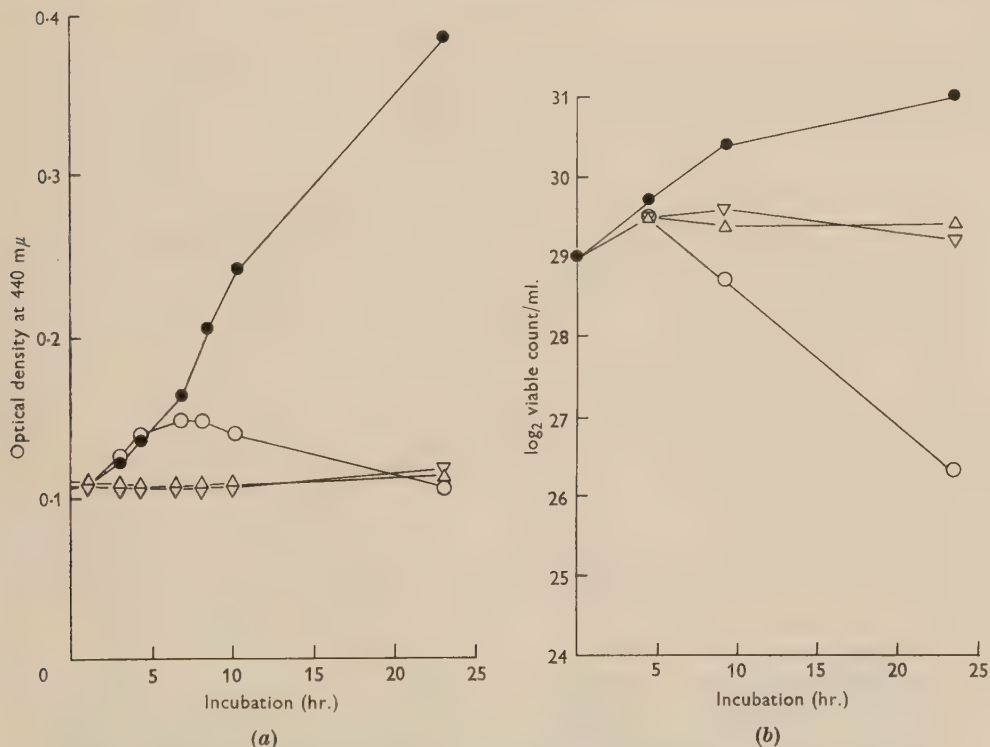


Fig. 1. The effect of the omission of glycerol and uracil on the turbidity and viability of cultures of *Mycoplasma mycoides*. (a) Turbidity; (b) viable element count. ●, Complete medium; ○, glycerol omitted; △, uracil omitted; ▽, glycerol and uracil omitted.

deficiency of uracil prevented death which would otherwise have occurred through glycerol deficiency, throughout this incubation period. Incubation in (ii) resulted in: an early increase, followed by a decrease, in turbidity (Fig. 1*a*); an early (after about 4 hr. of incubation) loss of streaming birefringence; later, an obvious increase in the viscosity of the culture, suggesting that extensive cellular lysis had occurred. Profound morphological changes culminating in lysis during incubation in the glycerol-deficient medium were evident in electron micrographs. Owing to technical difficulties, specimens for electron microscopy were obtained from a duplicate experiment on which turbidity measurements, but not viable counts, had been made. Electron micrographs prepared from cultures in (iii) (Pl. 1, fig. 3) showed a range of

forms characteristic of the organism grown in (i) (Pl. 1, figs. 1, 2) throughout the 24 hr. incubation period. In (ii) most of the forms were enlarged within 4–8 hr. of incubation; many had shrunken appendages, and a few flattened ghost-like forms were present (Pl. 1, fig. 4). After 24 hr. of incubation there was a great preponderance of ghost-like forms, most of which contained a single, small, electron-dense granule (Pl. 2, figs. 5, 6); there were only a few electron-dense forms which were probably representative of the remaining viable forms (Pl. 2, fig. 5). After 24 hr. incubation in (iv), the forms resembled preparations from (ii) after only 4 hr. incubation (Pl. 2, fig. 7). That is, a deficiency of uracil retarded the onset of the morphological changes caused by glycerol deficiency.

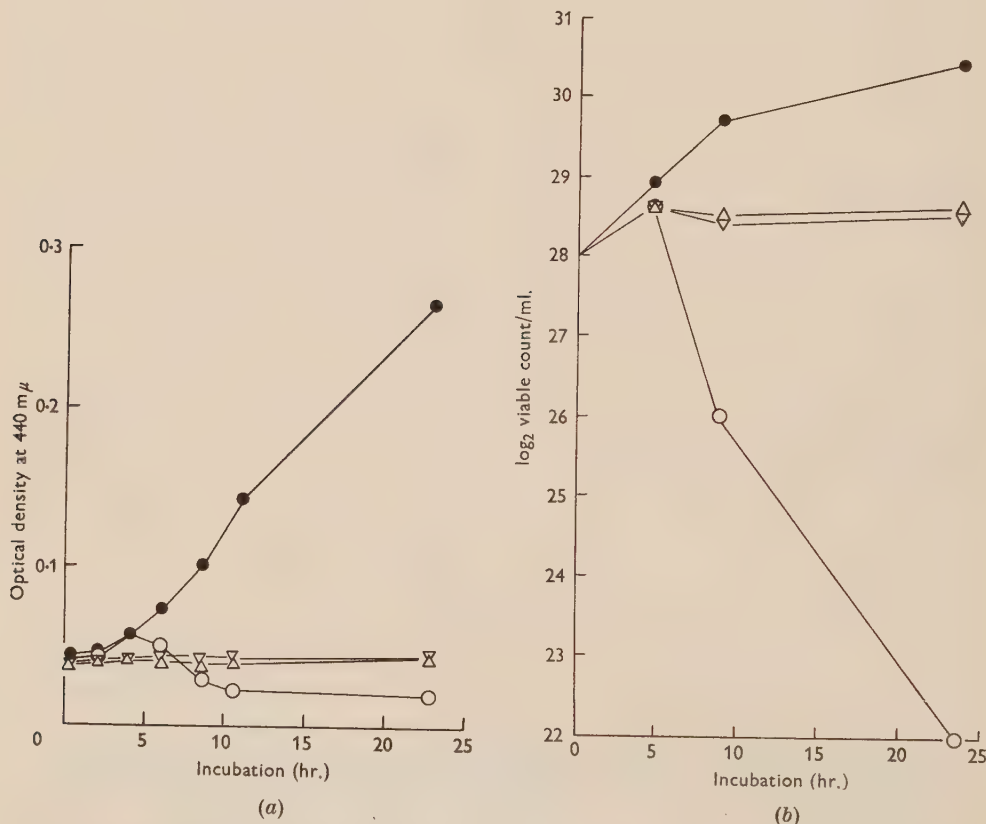


Fig. 2. The effect of the addition of chloramphenicol (CAP) on 'glycerol-less death' of *Mycoplasma mycoides*. (a) Turbidity; (b) viable element count. ●, Complete medium; ○, glycerol omitted; △, CAP (μg./ml.) added; ▽, glycerol omitted and CAP (20 μg./ml.) added.

Like the omission of uracil, the addition of chloramphenicol (CAP) prevented death caused by glycerol deficiency and the associated turbidity changes (Fig. 2a, b). Viable counts remained stationary during a 24 hr. incubation period in Medium A with the addition of 20 μg. CAP/ml., and in glycerol-deficient medium containing 20 μg. CAP/ml. Only about 1.5 % of the viable elements inoculated survived after 24 hr. of incubation in glycerol-deficient medium. There was a maximum mortality

rate of about 80 % per generation time. The greater death rate found in this experiment as compared with that illustrated in Fig. 1*b*, may have been due to differences in the size of the inocula used in the two experiments.

Effect of cholesterol + bovine serum Fraction C deficiency

Although direct evidence is lacking, it is thought that the function of bovine serum Fraction C is to bind cholesterol in a water-soluble assimilable form. Incubation of mycoplasma organisms in Medium A lacking both cholesterol and Fraction C resulted in an early loss (after about 5 hr. of incubation) of streaming birefringence.

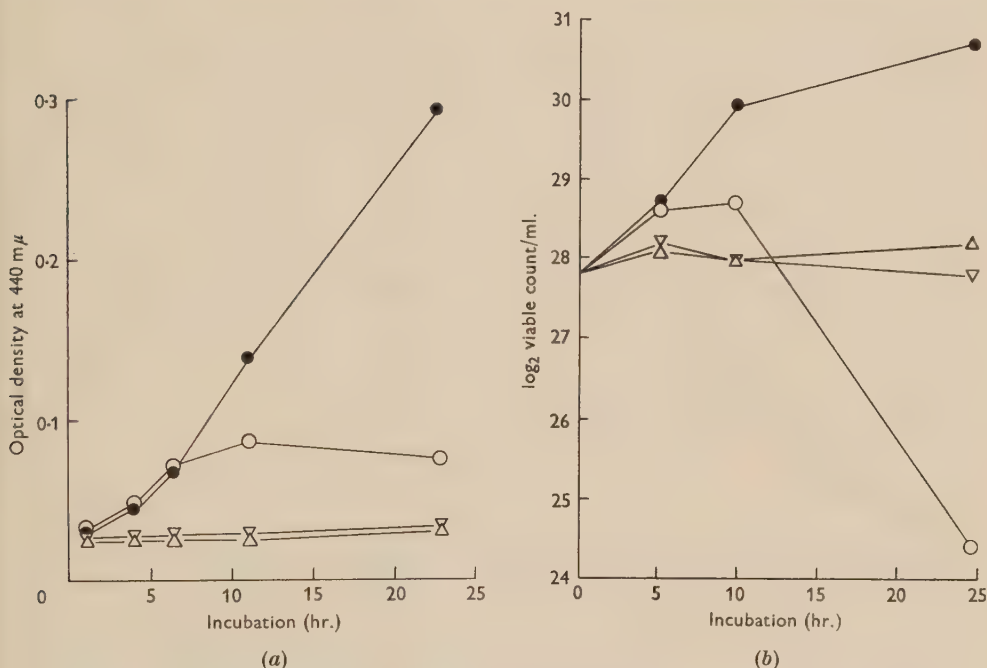


Fig. 3. The effect of the omission of cholesterol, bovine serum, Fraction C and uracil on the turbidity and viability of cultures of *Mycoplasma mycoides*. (a) Turbidity; (b) viable element count. ●, Complete medium; ○, cholesterol and bovine serum, Fraction C omitted; △, uracil omitted; ▽, cholesterol, bovine serum Fraction C and uracil omitted.

an increase followed by a gradual decrease in turbidity (Fig. 3*a*) and, after about 10 hr. of incubation, a rapid decrease in viability (Fig. 3*b*). As with glycerol deficiency, these changes did not occur when uracil was omitted. After 5 hr. of incubation many of the forms seen were swollen, and some had tenuous outgrowths which appeared to be in the process of becoming pinched off. A tendency for the forms to aggregate when the suspensions were dried on the grids was noticeable (Pl. 2, fig. 8). After longer incubation, the material appeared to be largely aggregated into masses in which little cellular structure could be seen (Pl. 3, figs. 9, 10). It is possible that a deficiency of cholesterol + bovine serum Fraction C caused the forms to become more susceptible to damage during the fixing and drying processes; nevertheless, the morphological changes differed from those caused by glycerol deficiency.

The omission of either bovine serum Fraction C or cholesterol singly caused decreases in viable counts, but the death rate was greater when both components were omitted (Fig. 4*a, b*).

Requirements for long-chain fatty acids

Medium A contains bovine serum albumin Fraction V. Tween 80 was also included, although it increased growth only slightly in the presence of Fraction V. Extraction with *iso*-octane + glacial acetic acid almost abolished the growth-promoting activity of Fraction V when Tween 80 was omitted. Its activity was partly restored

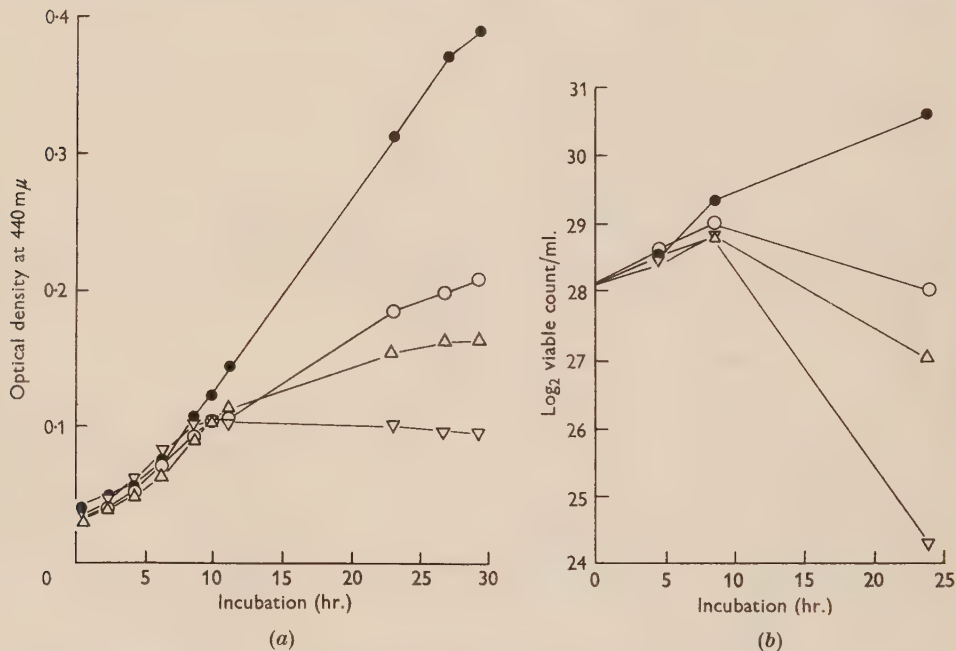


Fig. 4. The effect of the omission of cholesterol and/or of bovine serum Fraction C, on the turbidity and viability of cultures of *Mycoplasma mycoides*. (a) Turbidity; (b) viable element count. ●, Complete medium; ○, cholesterol omitted; △, bovine serum Fraction C omitted; ▽, cholesterol and bovine serum Fraction C omitted.

by the addition of Tween 80 or of oleate, and fully restored by a mixture of a saturated and an unsaturated fatty acid. Of the even-numbered saturated fatty acids from C₁₂ to C₁₈, palmitate and stearate were of about equal activity; laurate was less active when growth tests were performed in the presence of a constant concentration of oleate (Fig. 5). Myristate was as active as palmitate or stearate but the growth rate was slower (Fig. 6). The odd-numbered C₁₇ acid, margaric acid, was as active as palmitic or stearic acids (Fig. 7). Oleate, linoleate and linolenate were the only unsaturated fatty acids tested; oleate was the most active, the others being active only over a very narrow concentration range (Fig. 8).

The presence of *iso*-octane-extracted Fraction V was essential. The optimum concentration in the presence of 0.02 μmole each of palmitate and oleate/ml. was about 1.0 mg./ml.; growth was not significantly improved by increasing the concen-

trations of any of these components, or in the presence of more complex fatty acid mixtures.

All of these growth tests were performed in the presence of inositol, choline and leucovorin, although any influence these may have had on growth was not determined. Medium B was formulated as a result of these tests.

Cultures of *Mycoplasma mycoides* strain V5 in medium B showed very marked streaming birefringence, and electron micrographs showed a well developed branched mycelial morphology. It is now realized that Medium A is slightly deficient or disproportionate in its fatty acid composition (compare Pl. 1, fig. 1, and Pl. 3, fig. 11).

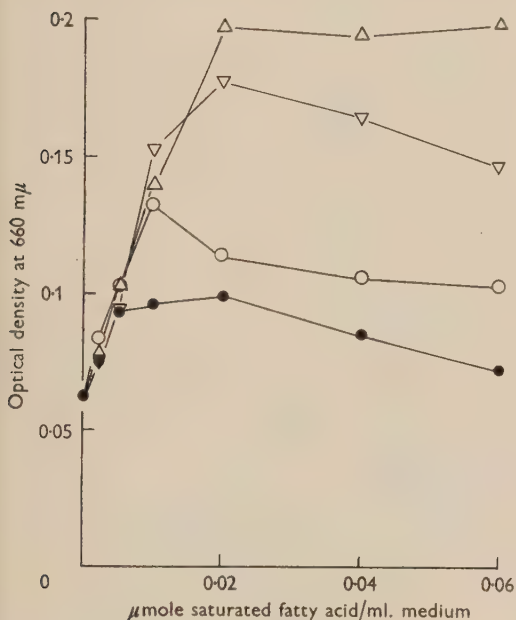


Fig. 5

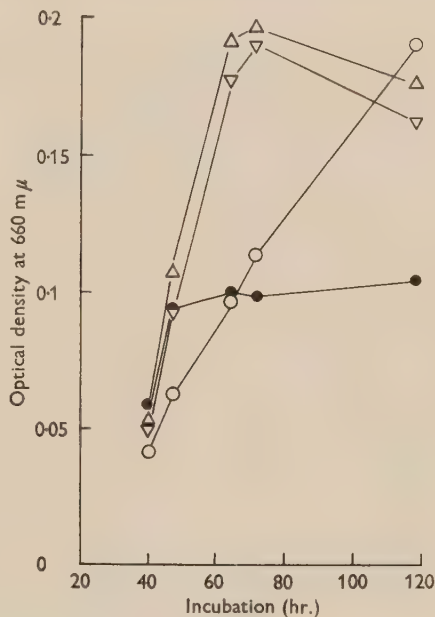


Fig. 6

Fig. 5. The effect of saturated fatty acids on growth of *Mycoplasma mycoides* in the presence of $0.01 \mu\text{mole}$ oleate/ml. Incubation period 71 hr. ●, Laurate added; ○, myristate added; △, palmitate added; ▽, stearate added.

Fig. 6. The effect of the C_{12} to C_{18} saturated fatty acids, in the presence of oleate, on the growth rate of *Mycoplasma mycoides*. Fatty acid concentration, $0.01 \mu\text{mole/ml}$. ●, Laurate; ○, myristate; △, palmitate; ▽, stearate.

Effect of fatty acid deficiencies

Replicate tubes of Medium B with the following omissions—(i) complete; (ii) without palmitate; (iii) without oleate; (iv) without palmitate and oleate; (v) without palmitate and uracil; (vi) without oleate and uracil; and (vii) without palmitate, oleate and uracil—were sown with 5.0×10^7 viable elements/ml. Viable counts and turbidity changes during incubation for 45 hr. are shown (Fig. 9*a, b*). The following points may be seen: (a) Without added palmitate there was a large increase in turbidity, the optical density after 45 hr. of incubation reaching a value about half of that of the cultures in complete medium; increases in the viable count almost equalled those in complete medium. (b) In the absence of oleate, there was an

increase, followed by a slow decrease, in turbidity, and, after about 15 hr. of incubation, a rapid decrease in viability. (c) In the absence of palmitate and oleate, the increase in turbidity was greater, and the decrease in viability less, than in the cultures deficient only in oleate. (d) The omission of uracil did not entirely prevent an increase in the number of viable elements when incubated in medium without added palmitate, nor did it prevent a decrease in the number of viable elements in media without oleate, or without oleate and palmitate. In two other experiments, however, uracil omission slowed the decrease in the number of viable elements in medium lacking both fatty acids.

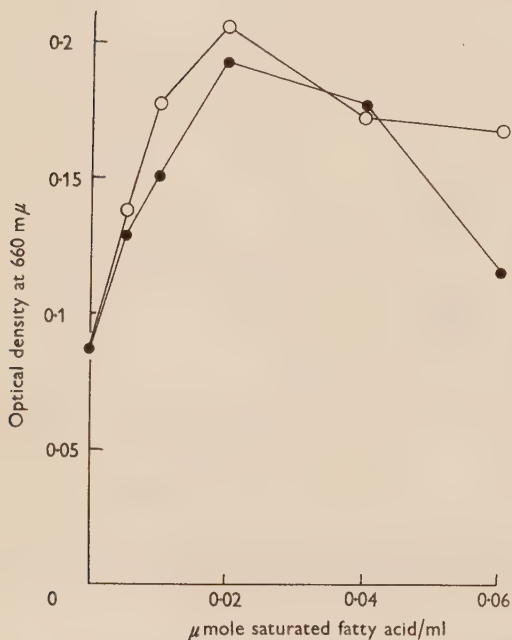


Fig. 7

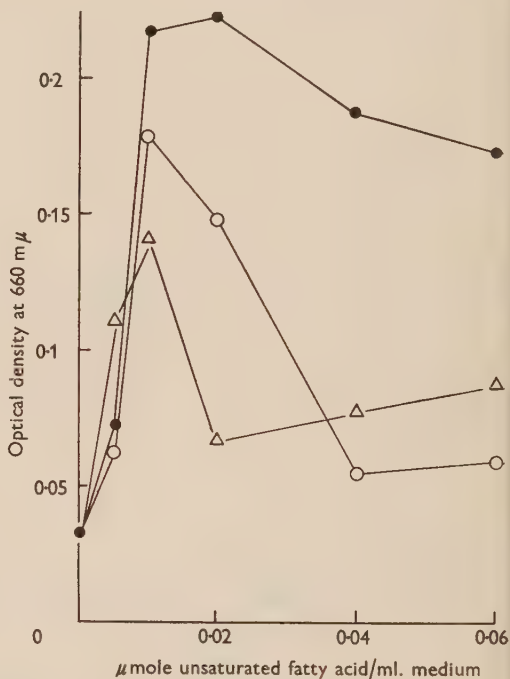


Fig. 8

Fig. 7. The effect of margarate and palmitate on growth of *Mycoplasma mycoides* in the presence of $0.01 \mu\text{mole}$ oleate/ml. Incubation period 70 hr. ●, Margarate added; ○, palmitate added.

Fig. 8. The effect of unsaturated fatty acids on the growth of *Mycoplasma mycoides* in the presence of $0.01 \mu\text{mole}$ palmitate/ml. Incubation period 71 hr. ●, Oleate added; ○, linoleate added; △, linolenate added.

Electron micrographs were made from a similar series of cultures, incubated for 20 hr., in which the turbidity changes closely paralleled those shown in Fig. 9a. The forms seen in growth in Medium B were highly branched tangled filaments of uniform thickness (Pl. 3, fig. 11). In Medium B from which palmitate had been omitted the forms were short plump pear-shaped or oval, and many had short outgrowths (Pl. 3, fig. 12). The forms in Medium B from which oleate had been omitted were of varied morphology (Pl. 4, fig. 13); flat disks, some with a thickened rim, and some with a number of bead-like structures ranged round the periphery, were all characteristic. The forms with the thickened rim, and those with the bead-like outgrowths,

doubtless correspond to the ring forms and to the asteroids, respectively, which have often been described in the past in morphological studies with the light microscope. An example of an asteroid is shown shadowed in Pl. 4, fig. 14, and after 'staining' with phosphotungstic acid in Pl. 4, fig. 15. The stain has penetrated the flat central portion of the form more readily than the bead-like outgrowths, as also in the ring form shown in Pl. 4, fig. 16. The form illustrated in Pl. 4, fig. 17, has five deeply stained areas (? vacuoles), some clearly defined lightly stained areas round part of the periphery, and a single lightly stained spot.

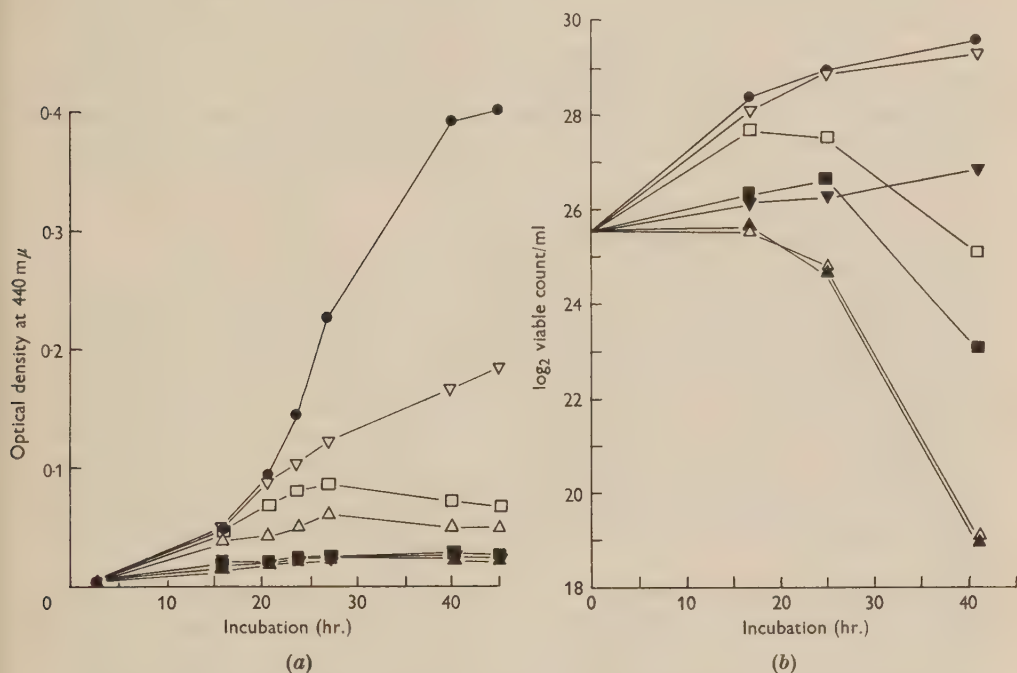


Fig. 9. The effect of the omission of palmitate, oleate and uracil on the turbidity and viability of cultures of *Mycoplasma mycoides*. (a) Turbidity; (b) viable element count. ●, Complete medium; △, oleate omitted; ▲, oleate and uracil omitted; ▽, palmitate omitted; ▼, palmitate and uracil omitted; □, palmitate and oleate omitted; ■, palmitate, oleate and uracil omitted.

DISCUSSION

The lipid precursor requirements for growth of *Mycoplasma mycoides* are complex. The observations suggest that a deficiency of glycerol, cholesterol, and probably other lipid precursors causes unbalanced growth, leading to the death of viable elements and lysis. When cytoplasmic synthesis was prevented (by uracil deprivation or by the addition of chloramphenicol) the death of viable elements and lysis were also prevented. The hypothesis is advanced that these nutrients are required for the synthesis of an undefined cell component which is necessary for the structural integrity of the cell, and that the synthesis of this is more sensitive to a deficiency of these nutrients than is the synthesis of cytoplasm. It is not excluded that the death of viable elements and lysis are secondary consequences of a primary defect elsewhere in the cell.

The effects of fatty acid deficiency require further investigation. The experiments were complicated by the probability that Medium B from which fatty acids were omitted was contaminated with suboptimal amounts of fatty acids derived from other medium constituents, e.g. from incompletely extracted bovine serum albumin Fraction V, or from the hydrolysed-casein preparations. Fatty acids may also have been carried over in significant amounts with the inoculum, because the fatty acid-dependence was found to vary with the size of the inoculum. It was necessary to diminish the size of the inoculum as compared with that used for the glycerol and cholesterol experiments, and to incubate for longer periods. Under these conditions, a deficiency of oleate caused decreases in counts of viable elements and lysis which were not prevented by uracil omission. Palmitate deficiency in the experiment described did not cause decreases in viable counts, but in other experiments in which incubation was continued still longer, such decreases were beginning to occur and might have been expected to be more rapid under conditions of greater deficiency. The highly branched filaments seen in Medium B may have contained many more 'nuclear equivalents' or *potential* colony-forming units than the observed viable count would indicate. This may explain why the viable count was no greater than in palmitate-deficient medium (Fig. 9*b*). The highly branched filaments would also have a higher surface/volume ratio than the plump elements seen in palmitate deficiency. The latter forms might therefore require less oleate to maintain their viability. The experiments suggest that the ratio of saturated to unsaturated fatty acids is an important factor. Shorb & Lund (1959) reported that a saturated and an unsaturated fatty acid, each inactive alone, were together required for the growth of two species of trichomonads; they also stressed the need for a proper balance of fatty acids. The growth-promoting activity of the C₁₇-acid, margaric acid, for *Mycoplasma mycoides* may indicate that this acid is incorporated into the lipids without alteration of chain length.

Knowledge of the limiting membrane of mycoplasma organisms might help to define their relations with other micro-organisms. Mycoplasma organisms appear to lack a rigid wall structure, and are known to lack the bacterial cell-wall 'mucocomplex' (Kandler & Zehender, 1957; Plackett, 1959). It may be suggested that the limiting membrane of *Mycoplasma mycoides* depends for its integrity on lipid components. Whether the galactan, described by Plackett & Buttery (1958) and Buttery & Plackett (1960), which accounts for about 10 % of the dry weight of the organism, and which also contains a lipid component, is associated with this structure, is not known.

The morphological changes which occur during incubation of mycoplasma forms in media deficient in glycerol, cholesterol, oleate and palmitate, appear to differ. An adequate concentration of fatty acids is needed for the development of branched filaments. The appearance of ring forms, asteroids, etc., often seen in cultures incubated for long periods in crude media, may be due to an unsaturated fatty acid deficiency.

The suggestion of a general loss of structure during incubation in medium deficient in the cholesterol + bovine serum Fraction C system is of interest because cholesterol is believed to be interspersed between the phospholipid residues in biological membranes, and to stabilize the structure. Butler & Knight (1960) described growth inhibition of *Mycoplasma laidlawii* by certain steroids, and its annulment by chol-

esterol. It would be of interest to know whether the growth-inhibitory steroids, cause unbalanced growth with subsequent death and lysis.

The nature of the compounds into which glycerol is incorporated is being studied. A substantial part is incorporated into a cardiolipin-like compound (Plackett, 1961). Glycerophosphate polymers of the teichoic acid type described in the cell-walls of certain species of bacteria, where they replace part or all of the ribitol phosphate (Armstrong, Baddiley & Buchanan, 1959), or large polymers of the type described by McCarty (1959), were not found. The glycerophosphate polymers forming part of the protoplast membrane of bacteria (Mitchell & Moyle, 1956; Gilby, Few & McQuillen, 1958) have not been characterized.

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EXPLANATION OF PLATES

Figs. 1-7 illustrate the morphological changes caused by incubation of *Mycoplasma mycoides* in medium deficient in glycerol and uracil; figs. 8-10 those resulting from deficiency in cholesterol and bovine serum Fraction C, and figs. 11-17 those from deficiency in palmitate and oleate. The specimens were photographed at a magnification of $\times 7200$. The final magnification of figs. 1-13 is $\times 14,400$ and that of figs. 15-17 is $\times 36,000$.

Figs. 1-4 and 6-8 were shadowed with gold-palladium; figs. 11-13 with gold-manganin and figs. 15-17 were stained with phosphotungstic acid.

PLATE 1

- Fig. 1. After 8 hr. incubation in Medium A.
 Fig. 2. After 24 hr. incubation in Medium A.
 Fig. 3. After 24 hr. incubation in Medium A, uracil omitted.
 Fig. 4. After 8 hr. incubation in Medium A, glycerol omitted.

PLATE 2

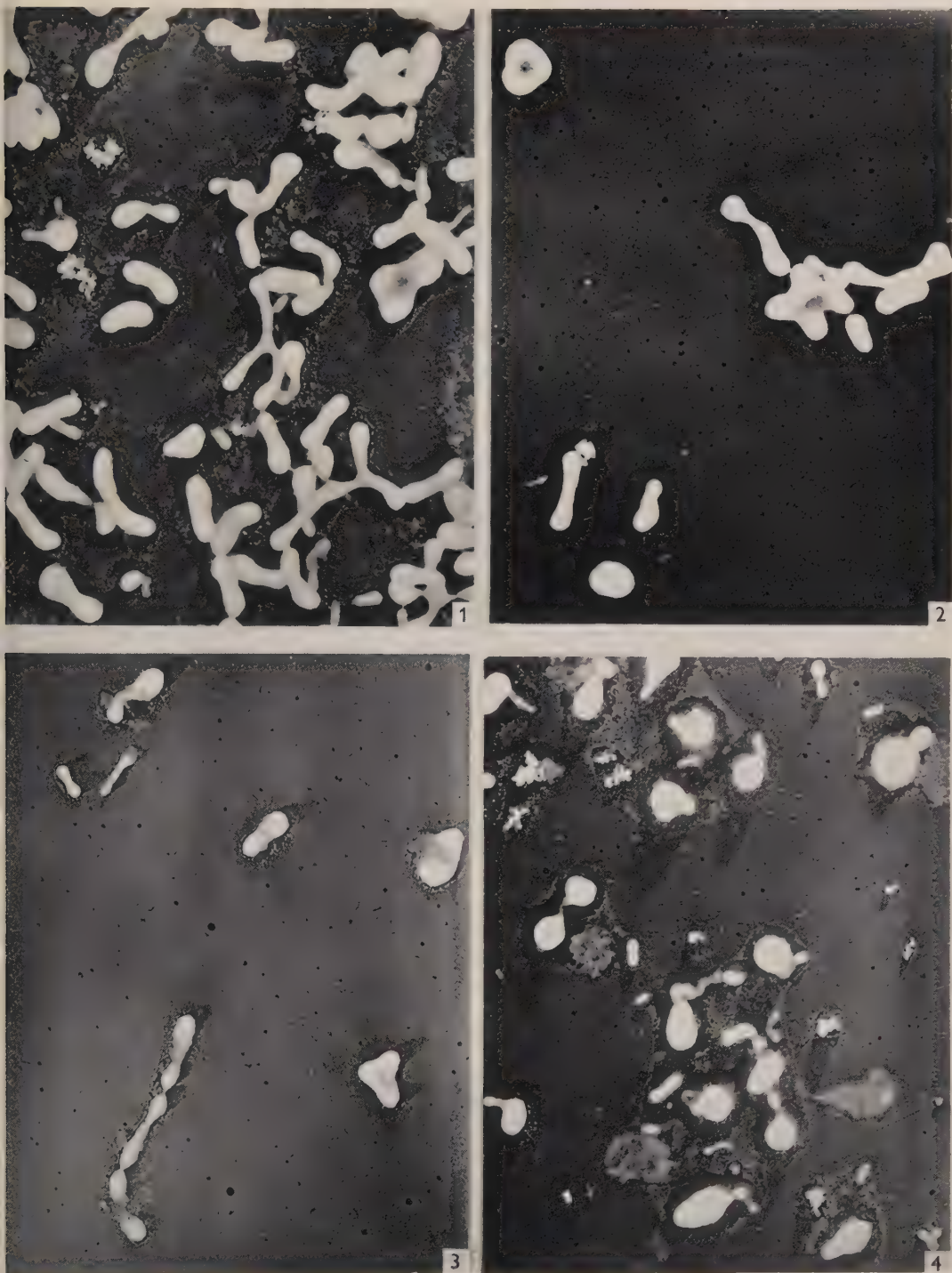
- Fig. 5. After 24 hr. incubation in Medium A, glycerol omitted; unshadowed.
 Fig. 6. As for fig. 5, shadowed.
 Fig. 7. After 24 hr. incubation in Medium A, glycerol and uracil omitted.
 Fig. 8. After 5 hr. incubation in Medium A, cholesterol and Fraction C omitted.

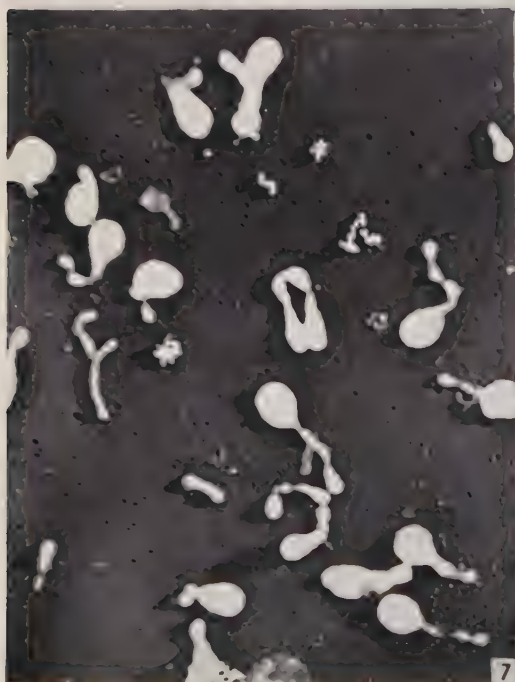
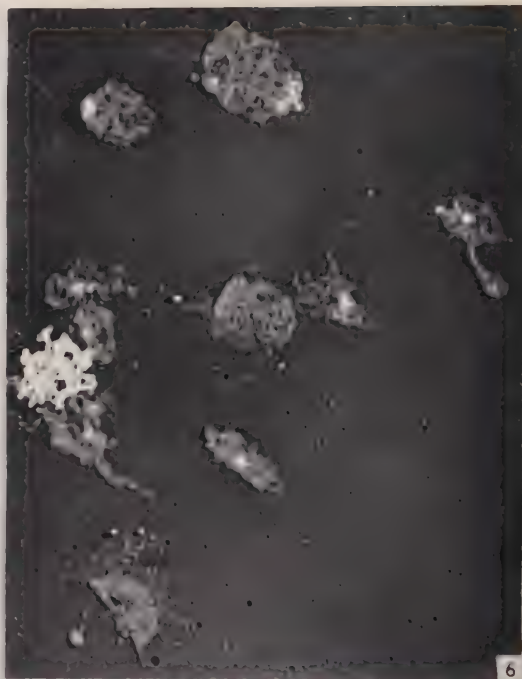
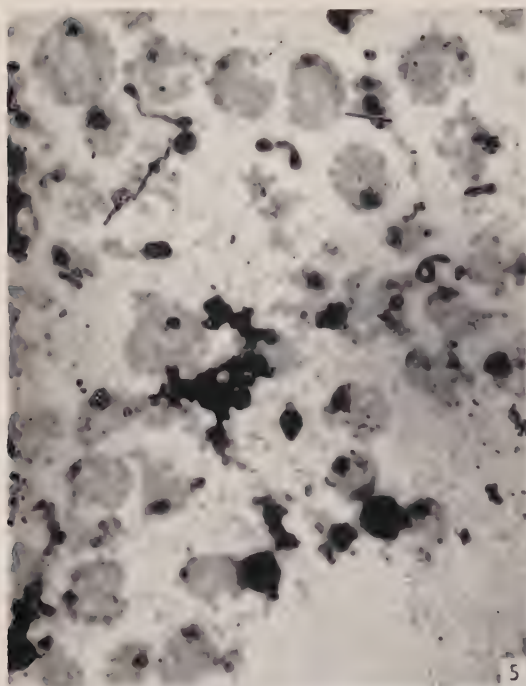
PLATE 3

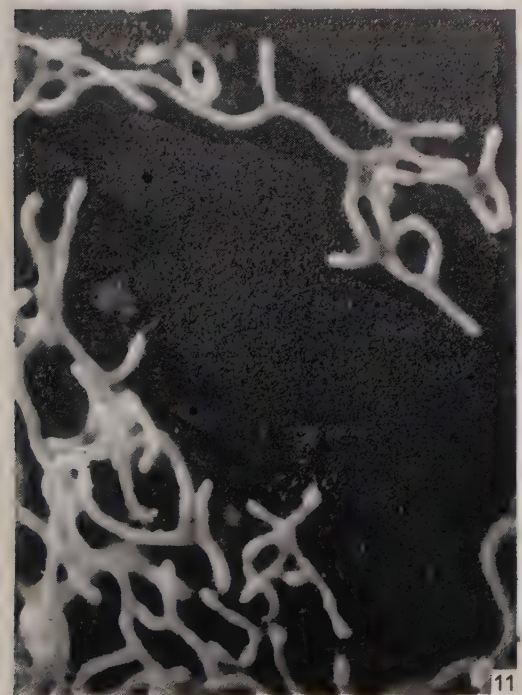
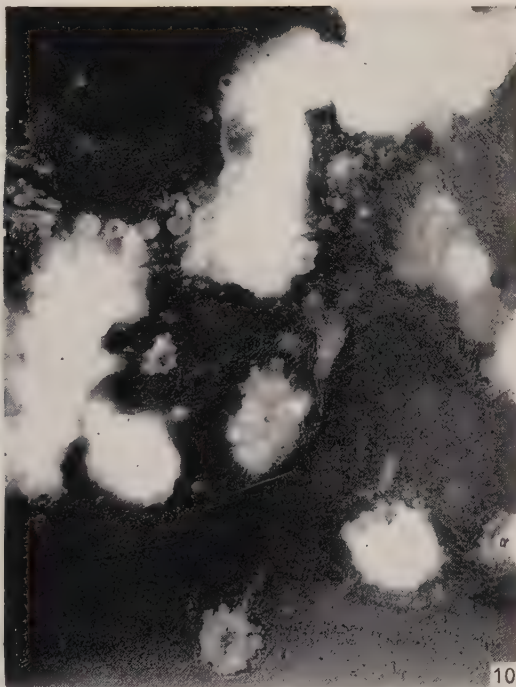
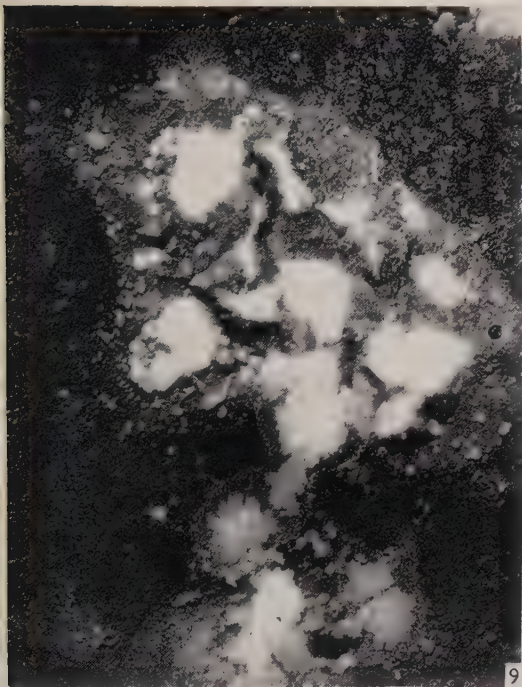
- Fig. 9. After 20 hr. incubation in Medium A, cholesterol and Fraction C omitted.
 Fig. 10. As for fig. 9, from another experiment.
 Fig. 11. After 20 hr. incubation in Medium B.
 Fig. 12. After 20 hr. incubation in Medium B, palmitate omitted.

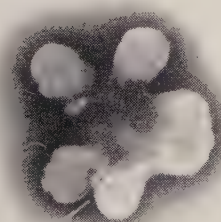
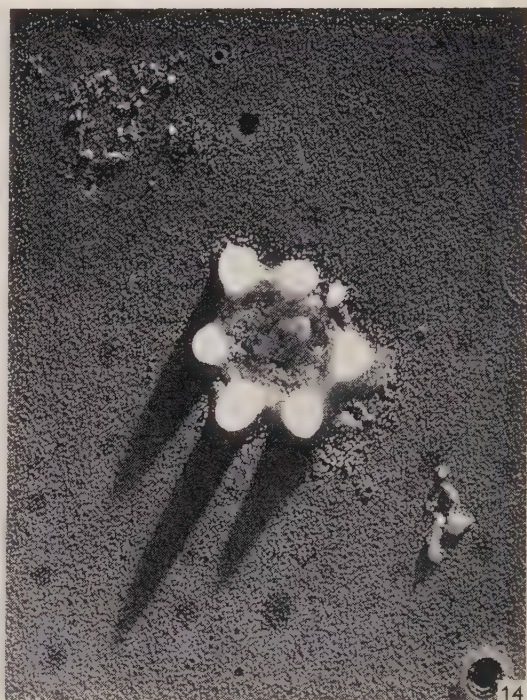
PLATE 4

- Fig. 13. After 20 hr. incubation in Medium B, oleate omitted.
 Fig. 14. As for fig. 13.
 Fig. 15. As for fig. 13.
 Fig. 16. As for fig. 13.
 Fig. 17. As for fig. 13.

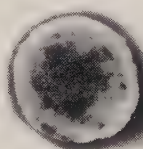








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Glyceride Hydrolysis and Glycerol Fermentation by Sheep Rumen Contents

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SUMMARY

Microbial hydrolysis of triglycerides was observed when these were incubated anaerobically at 37° with sheep rumen contents. The extent of hydrolysis was variable, but was often considerable (> 90 %) when linseed oil was used as substrate. The free fatty acids liberated were analysed by gas chromatography and, as compared with the acids present initially in glyceride combination, they were less unsaturated because of microbial hydrogenation. Linolenic acid was particularly effectively hydrogenated. No synthesis of long-chain fatty acids took place during the incubations and, apart from the possibility that in some experiments a limited conversion of stearic acid to palmitic acid took place, there was no evidence of significant degradation of long-chain acids. Glycerol liberated during the hydrolysis was completely metabolized, in part to volatile fatty acids, largely propionic acid. No mono- or diglycerides were detected as intermediates in the lipolysis of triglycerides. Analysis of the contents of the rumen, abomasum and small intestine of each of two slaughtered sheep, one of which had previously been fed on a diet rich in linseed oil, showed that most of the total higher fatty acids present in each of these three portions of the alimentary tract was in the form of free acids. It is concluded that microbial lipolysis results in the pre-digestion of much of the lipids ingested by the sheep as part of its feed.

INTRODUCTION

Attention was first directed to the influence of the rumen on lipids by Reiser (1951) who found a marked decrease in the linolenic acid content of linseed oil when it was incubated with sheep rumen contents. This effect was attributed to hydrogenation of double bonds by rumen micro-organisms and was substantiated by comparisons of the unsaturation of feed lipids with that of the lipids of rumen contents in sheep (Shorland, Weenink & Johns, 1955) and goats (Reiser & Reddy, 1956) and the detailed examination of the products of incubation of oleic, linoleic and linolenic acids with sheep rumen contents (Shorland, Weenink, Johns & McDonald, 1957). In all the foregoing studies the total lipid of rumen contents was saponified before determination of the unsaturation of the component fatty acids. In the present work, to examine more fully the effect of mixed rumen micro-organisms on esterified unsaturated fatty acids, several triglycerides were incubated with sheep rumen contents, and subsequently the lipids were extracted without previous saponification. These experiments, which resulted in the finding that

lipolysis could be effected by rumen organisms, are reported in the present paper, together with related observations on the nature of the lipids in different parts of the alimentary tract of the sheep. Preliminary accounts of some of these results have already been published (Garton, Hobson & Lough, 1958; Garton, Lough & Vioque, 1959; review by Garton, 1959).

METHODS

In vitro experiments

Incubation of glycerides with rumen contents. Samples of rumen contents were obtained from sheep with permanent rumen fistulas. The animals were fed on a mixture of hay and concentrates with free access to water and the samples (100–500 ml. as required) were taken 3–4 hr. after the last feed. After thorough mixing, portions (usually 100 ml.) were transferred to sterile 250 ml. conical flasks fitted with Bunsen valves. Each flask was placed up to its neck in a water bath maintained at 37°, and gassed with CO₂ to remove air. The pH value of the rumen contents was then determined by capillator (British Drug Houses, Ltd., Poole, Dorset) and adjusted when necessary to pH 6.5 by the dropwise addition of a sterile solution of Na₂CO₃ (25%, w/v). A weighed amount of glyceride, contained in a small glass cup, was then put into the rumen contents and the flask shaken; it was then flushed again with CO₂. At hourly intervals thereafter for 8 hr. the flask was shaken and adjusted to pH 6.5. The incubation was continued overnight for a further 16 hr. after which the pH value was recorded and the mixture acidified to pH 2.0 with 10N-H₂SO₄. Other flasks containing rumen contents only, or rumen contents which had previously been heated at 90° for 1 hr., were incubated at the same time and treated in the same way.

Extraction and fractionation of lipids. At the end of the incubation period 100 ml. ethanol were added to the acidified rumen contents and the mixture boiled under reflux for 10 min. After cooling, the aqueous ethanol was decanted and 200 ml. of a mixture of ethanol and ether (3+1, v/v) were added to the solid residue in the flask, which was then heated under reflux for 15 min. The ethanol+ether extract was filtered off and the solids remaining on the filter washed with warm ethanol+ether. The total ethanol+ether extracts were pooled with the aqueous ethanolic extract and this solution, containing the total lipid, was concentrated to 20–30 ml. by distillation on a water bath. To remove volatile fatty acids from the concentrated extract 50 ml. of a phosphate buffer (pH 6; containing 5.68 g. anhydrous Na₂HPO₄+49.0 g. anhydrous KH₂PO₄/l. water) were added and the mixture was extracted thrice with 50 ml. ether, the volatile acids remaining in the buffer solution as salts.

The combined ethereal extracts were shaken gently with 3×50 ml. 0.5% (w/v) aqueous KOH, followed by one washing of the ether with 50 ml. water; this treatment removed the free higher fatty acids as potassium soaps, leaving neutral lipids in solution in the ether. The solution of soaps was acidified dropwise with 10N-H₂SO₄ until no further precipitation of fatty acids took place. The acids were extracted thrice with 50 ml. ether and the combined ethereal extracts washed with water until free from mineral acid. The extract was then taken to dryness *in vacuo* in a tared flask to give the weight of free higher fatty acids, which were stored

under N_2 at $+1^\circ$ in the dark. After allowing for the weight of free fatty acids in the rumen contents alone, the extent of hydrolysis of a glyceride was derived from the weight of free fatty acids liberated expressed as % of the calculated weight of acids produced on complete hydrolysis of the glyceride. The slight increase in weight of the free acids resulting from the hydrogenation of unsaturated components was neglected.

In some experiments the alkali-extracted ethereal solution of neutral lipids was fractionated further; the solution was taken to dryness and saponified under reflux with excess 0.5N-ethanolic KOH. The saponified mixture was acidified dropwise with 10N- H_2SO_4 and the resultant mixture of fatty acids and unsaponifiable matter extracted with 3×50 ml. ether. From these combined extracts the fatty acids were recovered as described above for free fatty acids, leaving the unsaponifiable matter in the ether.

Incubation of glycerol with rumen contents. Following preliminary experiments in which glycerol was determined in rumen contents before and after the incubation with linseed oil, known amounts of free glycerol were incubated with rumen contents under the same conditions as those described for the incubation of glycerides. Glycerol was estimated by the method of Lambert & Neish (1950) following treatment of the rumen contents according to Johns (1953).

Fatty acid analyses. The free fatty acids resulting from lipolysis and, where appropriate, those derived from the residual neutral lipids, were converted to their corresponding methyl esters by refluxing with excess methanol containing 1% (w/w) H_2SO_4 . The recovered esters were analysed by gas chromatography at 170° using polymerised ethylene glycol adipate (prepared according to Farquhar *et al.* 1959) as liquid phase and argon as carrier gas. Appropriate model mixtures of fatty acid esters were chromatographed and the fatty acid composition of the samples from the incubations was calculated as described by Farquhar *et al.* (1959). The fatty acid composition of the glycerides used for incubation was determined in a similar way following saponification of the material with alkali and conversion of the acids to methyl esters.

Volatile fatty acids present in rumen contents before and after incubation with a triglyceride or glycerol were determined titrimetrically following acidification and steam-distillation of rumen contents (Friedemann, 1938); the mixtures of sodium salts of fatty acids so obtained were analysed qualitatively by paper chromatography (Elsden & Lewis, 1953).

Alimentary tract contents of slaughtered sheep

Samples of the contents of various parts of the alimentary tracts of two sheep were obtained immediately at slaughter which, for each animal, was 7 hr. after the last feed. One sheep had been fed for several months on a diet consisting largely of hay, linseed meal and maize to which 40 g. of linseed oil were added daily; the other had been fed on a mixture of concentrates including maize meal, groundnut meal, molassine meal, dried grass, oat dust and bruised oats. Each sample of alimentary tract contents was mixed with twice its weight of absolute methanol and filtered. The residue was retained and the filtrate was distilled to dryness on a water bath; the retained solids and the methanol-extracted matter were combined and extracted under reflux with excess chloroform + methanol mixture (2 + 1, v/v) for 15 min. The

mixture was filtered and the solids on the filter washed with hot chloroform + methanol, after which the combined filtrates were distilled to dryness to give the crude lipid extract. This material was dissolved in a convenient volume of ether before proceeding with fractionation into free fatty acids and neutral lipids as described above for incubated rumen contents.

RESULTS

Incubation of glycerides with rumen contents

In the first series of experiments 1.00 g. amounts of linseed oil were incubated with 100 ml. portions of whole rumen contents (in which the oil readily emulsified); after 24 hr. the free higher fatty acids were extracted and their iodine values determined. Hydrolysis usually resulted in the liberation of 60 % to more than 90 % of the esterified fatty acid residues of the original oil; occasionally the extent of hydrolysis was lower, in some experiments as low as 20 %. The results of two experiments showing considerable hydrolysis are given in Table 1. No lipolysis was observed in the flasks containing the rumen contents which had previously been heated (flasks 4 and 8) and the slight difference between the amounts of free acids recovered from flasks 3 and 4 and those from flasks 7 and 8 was due to traces of free acid present initially in the linseed oil. That hydrogenation of unsaturated acids had also taken place was indicated by the low iodine value of the recovered acids compared with the value (189.0) of the acids of the linseed oil itself; calculation gives the iodine value of the free acids derived from the oil as 121.4 (flask 2) and 112.2 (flask 6). Only traces of free glycerol were found in the rumen contents before incubation; following incubation in the presence or absence of linseed oil, none at all was detected.

Table 1. *Lipolytic activity of sheep rumen contents towards linseed oil*

Each flask contained 100 ml. rumen contents and, where indicated, 1.00 g. of linseed oil.

Flask	Treatment before incubation	Addition	Free fatty acids	
			Weight (mg.)	Iodine value
Experiment 1				
1	None	None	245	22.8
2	None	Linseed oil	859*	97.4
3	Heated at 90° for 1 hr.	None	236	31.6
4	Heated at 90° for 1 hr.	Linseed oil	268	44.7
Experiment 2				
5	None	None	291	29.2
6	None	Linseed oil	1144†	94.3
7	Heated at 90° for 1 hr.	None	253	41.3
8	Heated at 90° for 1 hr.	Linseed oil	311	50.0

* Representing about 62 % hydrolysis of the esterified fatty acids of the oil.

† Representing about 87 % hydrolysis of the esterified fatty acids of the oil.

In other experiments similar to those outlined in Table 1 linseed oil was incubated:
(i) with rumen contents from which almost all the micro-organisms had been

removed by centrifugation; (ii) with mixed sheep saliva (kindly collected for us by our colleague Dr R. N. B. Kay). In neither case was any hydrolysis of the oil observed. It was therefore concluded that lipolysis was probably due to the bacteria and/or protozoa of the rumen contents. When differential centrifugation was used to separate food particles and large protozoa from bacteria and small protozoa, almost all the lipolytic activity was found to be associated with the latter group of mixed organisms, which consisted mainly of bacteria. Attempts were made to prepare from mixed rumen organisms a cell-free extract which possessed lipase activity, but none was successful. The methods used included acetone-powders, grinding, shaking with ballotini beads, and rupture of the organisms with various detergents.

Further incubation experiments were carried out in which the free fatty acids resulting from the hydrolysis of linseed oil, olive oil and cocoa butter by whole rumen contents were analysed by gas chromatography. In addition, neutral lipids were extracted at the conclusion of the incubation period and volatile fatty acids were determined in the rumen contents incubated with and without the addition of triglyceride. Cocoa butter and olive oil did not form such stable emulsions in rumen contents as did linseed oil, and a few small globules of these triglycerides remained on the surface of the rumen contents throughout the incubation period.

The extent to which the esterified fatty acid residues in the glycerides were hydrolysed was about 95 %, 68 % and 40 %, respectively, for linseed oil, olive oil and cocoa butter. The combined weight of free fatty acids and neutral lipid recovered after the incubations showed almost quantitative recovery of the total weight of triglyceride added initially, due allowance being made for the amounts of lipid in the rumen contents *per se* and for the small amounts of glycerol liberated in the hydrolysis. The incubation of the glycerides resulted in a slightly enhanced formation of steam-volatile fatty acids as compared with the amounts found in rumen contents incubated under the same conditions without the addition of glycerides. This was probably due to some fermentation of liberated glycerol (see later). In Table 2 are shown the amounts and composition of the free fatty acids which resulted from hydrolysis of the glycerides, the fatty acid composition of the glycerides used, and the amounts and composition of the free acids present in the rumen contents alone. It is assumed that the free fatty acids extracted from the rumen contents alone were also present in the acids extracted from the glyceride incubations; Table 1 indicates that this is a reasonable assumption since a comparison of the amounts of free fatty acids in heated and unheated rumen contents (flasks 1 and 3 and flasks 5 and 7) showed that only very small amounts of free acids were produced during the incubation from lipids initially present in the rumen contents. Further, an even smaller amount might be expected to arise in this way when triglyceride was also present because of competition for enzyme by the additional substrate. Table 2 shows that, as compared with the composition of the fatty acids present initially in the triglycerides, the acids resulting from hydrolysis were extensively modified. Hydrogenation of the C₁₈ unsaturated fatty acids led to the production of enhanced proportions of stearic acid (cf. Shorland *et al.* 1957) and, in addition, palmitic acid appeared in increased proportions, particularly from linseed oil and olive oil. Small amounts of (as yet) unidentified acids were formed from linseed oil and olive oil, but not from cocoa butter. These acids, present in the

Table 2. *Composition and amounts of free fatty acids found after incubating sheep rumen contents with and without triglycerides and, for comparison, the fatty acid composition of the triglycerides*

Fatty acid	Expt. 3						Expt. 4		
	Acids from rumen contents alone (177 mg.)	Linseed oil		Olive oil		Acids from rumen contents alone (271 mg.)	Cocoa butter		
		Acids from incubation (940 mg.)*	Acids present in glycerides initially	Acids from incubation (659 mg.)*	Acids present in glycerides initially		Acids from incubation (353 mg.)*	Acids present in glycerides initially	
Composition (weight %)									
Unidentified ('acid A')	4.4	2.6	—	—	2.0	3.7	—	—	
Unidentified ('acid B')	5.4	2.4	—	—	1.7	5.8	—	—	
C ₁₆ saturated (palmitic)	17.9	13.8	5.6	10.3	27.2	20.5	27.4	24.5	
C ₁₆ mono-unsaturated	—	—	—	0.7	—	—	—	—	
C ₁₈ saturated (stearic)	57.5	31.7	5.8	2.9	26.7	61.5	46.9	34.1	
C ₁₈ mono-unsaturated	17.8	30.2	21.6	80.7	42.4	8.5	25.7	38.1	
C ₁₈ di-unsaturated	—	14.2	12.5	5.4	—	—	—	8.1	
C ₁₈ tri-unsaturated	—	5.1	54.5	—	—	—	—	—	

* Weight after subtraction of contribution of the free fatty acids from rumen contents alone.

free fatty acids of rumen contents *per se*, were not found in the dietary lipids of the sheep. On the gas chromatograms their methyl esters appeared in positions between methyl laurate and methyl tridecanoate, and between the latter and methyl myristate.

Whilst the studies of Shorland *et al.* (1957) showed that unsaturated fatty acids as such were hydrogenated when they were incubated with rumen contents, the experiments so far described here do not preclude the possibility that hydrogenation might take place whilst the acids were still in glyceride combination. Though in most of the experiments with linseed oil hydrolysis was almost complete, in others (as previously mentioned) the extent of hydrolysis was much less. Analysis of the neutral lipids remaining after partial (20 %) hydrolysis of 1.0 g. of linseed oil by 100 ml. rumen contents from one sheep showed that almost no hydrogenation of the esterified unsaturated acids had taken place. However, when rumen contents of a second sheep were used the neutral lipids remaining after 32 % hydrolysis of the oil did show changes in fatty acid composition though, as Table 3 shows, the free fatty acids were more extensively modified. In this particular experiment, in contrast to previous experiments with rumen contents of a different sheep (Table 2), hydrogenation did not result in enhanced amounts of stearic acid being produced (compare Wright, 1960), though much of the linolenic acid was converted to C₁₈ mono- and di-unsaturated acids; further, no additional palmitic acid was produced.

Table 3. *Composition of the free fatty acids and the fatty acids of the neutral lipids following partial hydrolysis of linseed oil and, for comparison, that of the fatty acids of the linseed oil glycerides*

Dash (—) indicates not detected.

Fatty acid	Source of acids		
	Free fatty acids*	Neutral lipids after incubation*	Linseed oil glycerides
	Composition (weight %)		
Unidentified	0.7	—	—
C ₁₆ saturated (palmitic)	5.7	5.3	5.7
C ₁₈ saturated (stearic)	4.0	5.2	4.2
C ₁₈ mono-unsaturated	24.4	18.8	15.6
C ₁₈ di-unsaturated	38.4	21.6	18.2
C ₁₈ tri-unsaturated	26.8	49.1	61.3

* Due allowance having been made for the contribution of the lipids in the rumen contents *per se*.

Incubation of glycerol with rumen contents

The hydrolysis of glycerides raises the question of the fate of the glycerol which is liberated. As already noted, no glycerol was detected in rumen contents following the almost complete hydrolysis of linseed oil. Similarly, when 0.5 g. amounts of glycerol were incubated anaerobically at 37° for 24 hr. with 100 ml. rumen contents (from a sheep fed on hay and concentrates) no glycerol was detectable after this time. In further experiments, 0.5–1.0 g. glycerol was incubated with 100 ml. rumen contents and small samples (1.0 ml.) were removed for glycerol determination at intervals up to 24 hr. after the start of the incubation. Within 2 hr. less than 80 %

of the initial amount of glycerol was present, within 4 hr. only about 50 % remained and the rest gradually disappeared within 24 hr. (Table 4). In no experiment did volatile fatty acids account for more than 50 % of the glycerol which was metabolized; the predominant acid produced was propionic acid.

Table 4. *Metabolism of glycerol in sheep rumen contents*

Glycerol (0.580 g.) was added to 100 ml. rumen contents.

Time after adding glycerol (hr.)	Recovery of added glycerol (%)
0	100
2	77.8
4	50.0
6	28.3
9	13.3
23	1.1
24	0

Possible intermediates in glyceride hydrolysis

In incubation experiments in which hydrolysis of linseed oil was less than 80 % after 24 hr. the neutral lipids were examined for the presence of possible intermediates of lipolysis, namely, mono- and di-glycerides. The chromatographic procedures of Borgström (1954), Barron & Hanahan (1958) and Hirsch & Ahrens (1958) all failed to reveal the presence of partial glycerides. Similar negative findings also resulted from analyses of the neutral lipids of samples of the incubation mixture taken at several intervals during the course of a 24 hr. incubation of linseed oil with rumen contents, although 'normal' hydrolysis progressively took place as evidenced by the increasing proportion of free fatty acids in the total lipids of the samples.

Table 5. *Free fatty acids in the alimentary tracts of two slaughtered sheep*

For diets of sheep, see Methods.

Source of digesta	Free fatty acids as % total fatty acids
Sheep 1	
Rumen	92
Abomasum	90
Small intestine (upper)	96
Small intestine (lower)	94
Sheep 2	
Rumen	78
Abomasum	94
Small intestine (total)	97

Alimentary tract contents

The proportion of free fatty acids in the total fatty acids obtained from the rumen, abomasum and small intestine of two sheep is given in Table 5. As described under Methods, sheep no. 1 was given the diet containing linseed oil and sheep

no. 2 was fed largely on concentrates. The greater part of the total fatty acids of the alimentary tract contents of both animals was in the form of free higher fatty acids; gas chromatographic analysis of those derived from sheep no. 2 showed that stearic acid, palmitic acid and C_{18} mono-unsaturated acid were the major components of the acids in each of the three parts of the tract which were examined (compare the free fatty acids of rumen contents given in Table 2).

DISCUSSION

Though it has been known for some time that carbohydrates and proteins undergo hydrolytic and fermentative changes in the rumen, little attention has hitherto been paid to the fate of lipids which constitute as much as 5–10% of the dry weight of many common feedingstuffs. As outlined in the Introduction, it has been established within the last decade that centres of unsaturation of fatty acids can be hydrogenated in the rumen, apparently under the influence of the micro-organisms present. The findings of the present work now show that the micro-organisms can effect considerable hydrolysis of fatty acids from glyceride combination and, since the preliminary report of these studies appeared (Garton *et al.* 1958), Dawson (1959) has found that phospholipids too can be hydrolysed to their components (i.e. fatty acids, glycerol and a nitrogenous base) by sheep rumen organisms. Thus ingested feed lipids can be pre-digested in the rumen and most of the higher fatty acids in the digesta leaving the rumen are, as Table 5 shows, in the free state. This is a situation markedly different from that in simple-stomached animals in which ingested lipids are not subject to hydrolysis until they reach the small intestine.

It is concluded that rumen bacteria produce a lipase and, as the following paper (Hobson & Mann, 1961) describes, lipolytic bacteria have been isolated from sheep rumen contents. Since no enzyme activity was detected in rumen contents except in the presence of micro-organisms, the lipolysis must take place either within the bacteria or at the point of contact between bacterium and lipid particle. No mono- or di-glycerides were detected during the lipolysis of triglyceride, indicating that, if formed at all, these possible intermediates have a very transient existence. Indeed, it appears that the enzyme system is non-specific in its activity towards fatty acids occupying the α - and β -positions on the glycerol molecule; in this respect the system seems to resemble lipoprotein lipase rather than pancreatic lipase (compare Korn, 1960).

The hydrogenation of the unsaturated fatty acids of triglycerides apparently takes place more rapidly when they are split off rather than when they are in glyceride combination. It should be mentioned that hydrogenation results in the production of spatial and positional isomers of unsaturated acids (Shorland *et al.* 1957); these are included, where appropriate, in Tables 2 and 3 under the heading ' C_{18} mono-, di- and tri-unsaturated acids'. Linolenic acid forms a very large proportion of the total fatty acids of pasture grasses (Garton, 1960). Our studies have confirmed the observations of Shorland *et al.* (1957) that this acid is particularly effectively hydrogenated. The micro-organisms responsible for hydrogenating double bonds and the source of the necessary hydrogen have not yet been investigated, though Wright (1959, 1960) has shown that, in addition to bacteria, protozoa are probably involved.

In the experiments illustrated in Table 2 some additional palmitic acid was apparently formed in some way at the expense of the C_{18} fatty acids of linseed oil, olive oil and cocoa butter. Though no significantly increased amounts of lower volatile fatty acids were produced during these incubations, it seems possible that the palmitic acid was formed from stearic acid. This question forms part of a current investigation using synthetic glycerides containing ^{14}C -labelled stearic acid. In connexion with the occurrence of free higher fatty acids in rumen lipids, it may be noted that there is no evidence that they can be synthesized in significant amounts *de novo* in the rumen other than in the formation of the lipids within the micro-organisms themselves. Gray, Pilgrim, Rodda & Weller (1952) showed that when ^{14}C -labelled acetate or ^{14}C -labelled propionate were incubated with sheep rumen contents, butyrate arose by condensation of acetate units, and valerate by condensation of acetate and propionate units; no significant synthesis of longer-chain acids was observed.

Johns (1953) showed that the fermentation of free glycerol by sheep rumen contents *in vitro* and *in vivo* led to the formation of propionic acid. That propionic acid is the principal volatile fatty acid produced was confirmed in the present work, though no more than 50 % of the glycerol which was metabolized could be accounted for on a 'carbon balance' basis; the fate of the remainder has still to be investigated. As described in the following paper (Hobson & Mann, 1961), relatively more of the glycerol carbon was accounted for as volatile fatty acids (largely propionic) when glycerol-fermenting organisms from sheep rumen were grown in media containing glycerol or glycerol + acetate.

Triglycerides, though present in the lipids of concentrates made from seeds (e.g. linseed meal, maize meal, groundnut meal), do not form a very high proportion of the lipids of such feeds as grasses and clover in which galactosyl glyceryl esters of fatty acids predominate (Weenink, 1959; Garton, 1960). Though the metabolism of these esters by rumen micro-organisms has not yet been studied in detail, preliminary experiments indicate that they are readily hydrolysed.

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The Isolation of Glycerol-Fermenting and Lipolytic Bacteria from the Rumen of the Sheep

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SUMMARY

Facultatively anaerobic bacteria appear to play little part in glycerol fermentation in the sheep rumen. Amongst the most important members of the glycerol-fermenting flora are strict anaerobes of the group *Selenomonas ruminantium* var. *lactilyticas*. Three isolates of a different group of strictly anaerobic curved Gram-negative rods, which hydrolyse linseed oil and tributyrin as well as fermenting glycerol, were obtained in numbers which suggest they are amongst the more important lipolytic bacteria in the rumen. The main product of fermentation of glycerol by the selenomonads and the lipolytic bacteria is propionic acid, but other acids are also formed. The properties of these bacteria and some others isolated during the experiments are described. All the bacteria appear to be normal inhabitants of the rumen of sheep fed a number of different rations. The numbers of these organisms were not appreciably increased by feeding glycerol or triglycerides to the animals.

INTRODUCTION

Over the last few years a number of papers have been published about the effects of dietary fats and oils on the digestibility of foodstuff components. Also, the hydrogenation of unsaturated fatty acids in the rumen has been investigated by a number of workers (see review by Garton, 1960). During investigations on the nitrogen metabolism of ruminants Dr M. I. Chalmers of this Institute found that the addition of oils or fats to sheep rations had a marked effect on the ruminal ammonia concentrations. The separate components of these triglycerides (fatty acids, glycerol) also affected ammonia concentrations as might be expected, since it has been shown that triglycerides of long-chain fatty acids can be rapidly hydrolysed by mixed rumen organisms (Garton, Hobson & Lough, 1958; Garton, Lough & Vioque, 1961). The work described here was undertaken to see whether some of the bacteria presumed to be responsible for the hydrolysis of dietary glycerides and the fermentation of the glycerol formed could be isolated and studied in more detail. Also to be examined was the question whether such bacteria were an intrinsic part of the normal rumen flora, or whether they increased in numbers (or differed in types) in the rumens of sheep being fed different amounts of glycerides or glycerol. Since the ruminal ammonia concentrations were altered by feeding these substances it was of interest to see whether glycerol-fermenting or lipolytic bacteria utilized or produced any large amount of ammonia during growth. A number of glycerol-containing media were used in an endeavour to isolate not only strictly anaerobic rumen bacteria, but also facultative anaerobes and bacteria which do not need the exacting conditions of

some rumen bacteria (see review by Bryant, 1959). Acetate was included in some of the media in an attempt to isolate bacteria which fermented glycerol and acetate to butyric acid (compare *Clostridium tyrobutyricum*) since the experiments with whole rumen contents suggested that propionic acid was not the only end product of glycerol fermentation in the rumen as suggested by Johns (1953), and acetate is a normal constituent of rumen fluid. Linseed oil was chosen as substrate for the lipolytic bacteria since it was being fed to some of the sheep in linseed cake and as added oil; further, the component fatty acids of the glycerides of linseed oil are similar to those of total grass lipids.

METHODS

Media

Some of the important bacteria isolated from the rumen will not grow under the conditions usually used for growth of 'strict anaerobes' (for example clostridia). Special techniques for the isolation of these bacteria have been devised and these are briefly outlined below. In addition to a low E_h and CO_2 some rumen bacteria need growth factors found in rumen fluid, and this is often included in media for isolation of rumen bacteria.

General methods. The technique used for the preparation of the media specified as 'incubated under CO_2 ' (no. 4-14) was based on that originated by Hungate (1950). In this method the various solutions making up the media are saturated with CO_2 and mixed under a stream of oxygen-free CO_2 , and contain a reducing agent which brings the E_h below that at which resazurin is colourless. The oxidation-reduction potential of the media described in this paper, measured between a calomel and a platinum electrode, was *c.* -350 mV. Small amounts of these media (*c.* 10 ml.) were dispensed into 6 in. \times $\frac{5}{8}$ in. tubes under a stream of CO_2 and tightly stoppered with rubber bungs. Larger volumes of media were prepared in rubber-stoppered bottles using a similar technique. All inoculations of this type of medium were carried out under a stream of CO_2 . Any media showing a trace of pink oxidized resazurin were discarded as the strictly anaerobic rumen bacteria will not grow under such conditions.

General constituents of media. Mineral solution (a) contained (g./l.): KH_2PO_4 , 3.0; $(\text{NH}_4)_2\text{SO}_4$, 6.0; NaCl, 6.0; MgSO_4 , 0.6; CaCl_2 , 0.6. Mineral solution (b) contained (g./l.): K_2HPO_4 , 3.0. Rumen fluid was prepared by straining rumen contents (freshly obtained from a hay-fed sheep) through gauze and centrifuging at 62,000*g* for 10 min. The clear liquid was kept for not more than a few days at 2° before use.

Media used for glycerol-fermenting bacteria

Media 1 to 3 were used for the isolation of rumen bacteria less exacting in requirements for a low E_h and presence of CO_2 . *Medium 1* contained, per 100 ml.: Bactocasitone, 1.5 g.; Difco yeast extract, 0.5 g.; NaCl, 0.5 g.; L-cystine, 0.075 g.; thio-glycollic (mercaptoacetic) acid, 0.03 ml.; agar, 0.075 g.; glycerol, 1 ml.; bromocresol purple, 0.1 ml. of 1.6% (w/v) ethanolic solution; water to 100 ml. All the constituents except the glycerol, which was added as a sterile filtered solution, were autoclaved together at 120° for 15 min. The medium was dispensed in 9 ml. amounts in tubes plugged with cotton wool and tenfold dilutions of rumen contents prepared in this medium. Incubation was in air. *Medium 2.* This medium was like medium 1, but contained agar 2 g., and was incubated in plates in a McIntosh & Fildes anaero-

bic jar under hydrogen. *Medium 3* contained: mineral solution (a), 15 ml.; mineral solution (b), 15 ml.; rumen fluid, 20 ml.; yeast extract, 0.25 g.; glycerol, 1 ml.; agar, 2 g.; water, 49 ml.; bromocresol purple, 0.1 ml. of 1.6% (w/v) ethanolic solution. All the constituents except glycerol, which was added as a filtered solution, were autoclaved together. This medium was incubated in plates in a McIntosh & Fildes anaerobic jar under hydrogen, except where noted.

Media 4 to 14 were used for the isolation or testing of rumen bacteria exacting in requirements for a low E_h and CO_2 , and were prepared as described under General Methods and incubated under CO_2 . *Medium 4* contained mineral solution (a), 15 ml.; mineral solution (b), 15 ml.; rumen fluid, 40 ml.; Difco yeast extract, 0.25 g.; water, 29 ml.; bromocresol purple, 0.1 ml. of 1.6% (w/v) ethanolic solution; glycerol, 1 ml.; sodium acetate, hydrated, 1.49 g.; NaHCO_3 , 0.4 g.; cysteine HCl, 0.05 g.; pH 6.8. The mineral solutions, rumen fluid, yeast extract, indicator and water were autoclaved together at 120° for 15 min. Immediately on removing from the autoclave oxygen-free CO_2 was bubbled through the solution and the flask stoppered. The glycerol, acetate, bicarbonate and cysteine were then added as a sterile filtered solution, and the medium dispensed in 9 ml. amounts. Tenfold dilutions of rumen contents were usually made directly into this medium. *Medium 5*. This medium was like medium 4 except that bromocresol purple was omitted and agar (2%, w/v) and resazurin (0.0001%, w/v) were added. *Medium 6* was like medium 5, but without agar. *Medium 7* was like medium 4, except that sodium acetate was omitted and the glycerol was replaced by the appropriate 'sugar' to 0.5% or 1% (w/v). *Medium 8* contained per 100 ml.: mineral solution (a), 15 ml.; mineral solution (b), 15 ml.; rumen fluid, 20 ml.; water, 49 ml.; NaHCO_3 , 0.4 g.; glycerol, 1 ml.; cysteine HCl, 0.05 g.; resazurin, 0.1 ml. of 0.1% (w/v) solution. *Medium 9* was like medium 8 but contained in addition yeast extract, 0.25 g. After incubation for 2 days ammonia in inoculated and uninoculated media was determined by the method of Conway (1957). The lipolytic bacteria were also tested in media 8 and 9.

Media used for lipolytic bacteria

These media were prepared and incubated under CO_2 as described under General Methods. To prevent alteration of the linseed oil by heating, the oil was taken from the lower part of a deep bottle kept at 2° and added to media without sterilization. No bacteria were ever found to grow from such samples of oil placed in uninoculated media. Saliva was used as an emulsifying agent for the linseed oil.

Saliva-based medium. *Medium 10* contained, per 100 ml.: glucose, 0.2 g.; yeast extract, 0.5 g.; NaCl, 0.5 g.; cysteine HCl, 0.1 g.; sheep parotid saliva, 99 ml.; linseed oil, 1 ml.; resazurin, 0.1 ml. of 0.1% (w/v) solution; K_2HPO_4 , 0.087 g.; KH_2PO_4 , 1.29 g.; final pH 6.9. The phosphates, yeast, NaCl, cysteine and resazurin were mixed in 95 ml. saliva, gassed with CO_2 and heated in boiling water until the dye was reduced. The bottle was then stoppered tightly under CO_2 and autoclaved at 120° for 15 min. The linseed oil was then added as an emulsion and the glucose as a concentrated filtered solution in amounts sufficient to bring the constitution of the medium to that given above. The complete medium was then mechanically shaken to produce an even emulsion. *Medium 10* was dispensed in 9 ml. amounts and inoculated with 1 ml. portions of tenfold dilutions of rumen contents made in a solution of phos-

phates, cysteine and resazurin in saliva in the concentrations used in the medium. The dilution blanks were autoclaved under CO_2 and dilutions made in an apparatus enabling CO_2 to be continuously bubbled through the blanks.

Rumen fluid-based media (11 to 13). Medium 11 contained, per 100 ml.: mineral solution (a), 15 ml.; mineral solution (b), 15 ml.; rumen fluid, 40 ml.; water, 29 ml.; resazurin, 0.1 ml. of 0.1 % (w/v) solution; NaHCO_3 , 0.4 g.; cysteine HCl, 0.05 g.; glucose, 0.1 g.; linseed oil, 1 ml. The minerals, rumen fluid, water and resazurin were autoclaved together at 120° for 15 min. The NaHCO_3 , cysteine and glucose were added as a sterile filtered aqueous solution, and the linseed oil as a 50 % (v/v) emulsion in sterile rumen fluid. The completed medium no. 11 was shaken under CO_2 to emulsify the oil and then dispensed in 9 ml. amounts. Tenfold dilutions of rumen contents were made under CO_2 in a sterile solution of minerals (a) + (b), cysteine and resazurin in the percentage proportions used in the medium. Portions (1 ml.) of each dilution were added to 9 ml. medium. Medium 12 was like medium 11 except that glucose was omitted. Medium 13 was like medium 12 but containing 2 % (w/v) agar. The linseed oil emulsion was added to medium 13 at a temperature of about 70° and it was shaken immediately so that the agar did not set whilst the oil was being emulsified. Medium 13 was dispensed and kept at 50° until inoculated, after which the tubes were rolled under cold water; dilutions of rumen contents were made directly in this medium. Medium 13 + 1 % (v/v) tributyrin in place of oil was used to test for esterase activity.

Casitone + yeast extract-based medium. Medium 14 was like medium 12 except that the rumen fluid was replaced by Bacto-casitone (1.5 g.) and yeast extract (0.25 g.).

Properties of glycerol-fermenting bacteria

Biochemical tests on selenomonads. Hydrogen sulphide was tested for in medium 6 by lead acetate paper, gelatin liquefaction in the same medium with sterile gelatin + charcoal tablet added (Kohn, 1953), and nitrate reduction in this medium with 0.2 % (w/v) KNO_3 added. Indole and Voges-Proskauer tests were made in a medium similar to medium 6 but with 0.5 % (w/v) glucose in place of glycerol, Bacto-casitone added to 1 % (w/v) and the rumen fluid decreased to 10 % (v/v). Products of glycerol fermentation were determined in medium 6 with rumen fluid decreased to 10 % (v/v). Fermentation reactions were tested in medium 7.

The properties of other bacteria. The fermentation reactions of the *Clostridium* and *Aerobacter* spp. were tested in peptone water and other reactions by the methods given in *Manual of Microbiological Methods* (1957). Products of glycerol fermentation by the clostridia were determined in cultures grown in medium 1.

Motility tests were made on selenomonad cultures grown 17 hr. in medium 6 + 0.1 % (w/v) glucose replacing glycerol. Organisms were examined in sealed capillary tubes and stained for flagella by Liefson's method (*Manual of Microbiological Methods*, 1957).

Properties of lipolytic bacteria

Biochemical tests. Fermentation reactions were tested in medium 12 or in medium 14 (without oil) with the 'sugar' at 1 % or 0.5 % (w/v) concentration and bromocresol purple added. Hydrogen sulphide and indole production were tested in medium 14 without oil but with glycerol, nitrate reduction in the same medium with

0.1 % (w/v) KNO_3 added, and gelatin hydrolysis also in this medium with a gelatin + charcoal tablet added.

Motility tests were made on cultures grown 17 hr. in medium 14 + 0.1 % (v/v) glycerol in place of oil. Organisms were examined in wet preparation by dark-ground illumination and stained for flagella by Liefson's method (*Manual of Microbiological Methods*, 1957).

General Methods

Determination of lipolytic activity. The whole culture, or uninoculated medium blank (10 ml.) was brought to pH 5.6–5.8 by addition of 2N-HCl and extracted with 3×10 ml. portions of ether. The ether extract was then washed with successive 10 ml. portions of water saturated with ether until the washings were at pH 6.5–7. The ether was removed by distillation (for the last few minutes *in vacuo*) the oily residue dissolved in 10 ml. 95 % (v/v) ethanol in water and titrated with 0.05N-NaOH (phenol red as indicator). In preliminary experiments the results obtained by this method were checked against those obtained by extraction and weighing of the free fatty acids by the methods given in the previous paper (Garton *et al.* 1961).

Determination of fermentation products. Volatile fatty acids were steam-distilled from the acidified medium after removal of cells (Friedemann, 1938) and analysed qualitatively by paper chromatography (Elsden & Lewis, 1953) or quantitatively by chromatography on celite columns (Bueding & Yale, 1951). Lactic acid and formic acid were determined colorimetrically (Barker & Summerson, 1941; Grant, 1947) and succinic acid by a manometric method (Umbreit, Burris & Stauffer, 1957). Glycerol was determined by the method of Lambert & Neish (1950).

Stock cultures. Stock cultures of the clostridia were kept in Robertson's cooked meat medium and the *Aerobacter* strains on nutrient agar slopes. The selenomonads were kept initially at -20° on slopes of medium 5, and subcultured every 10–14 days. Later slopes stored at 2° were found to keep just as effectively. Cultures were also freeze-dried in 7.5 % (w/v) glucose serum broth and in 10 % whole milk. These dried cultures kept at 2° could be revived after 3 months. The lipolytic organisms were maintained at 2° on slopes of medium 14 with agar + glycerol in place of oil, transferred every 4 weeks. Cultures freeze-dried as previously described did not revive.

Sheep rations and the collection of rumen samples. The basal ration consisted of a mixture of linseed cake meal, casein and a concentrate mixture (ground maize, crushed oats, bran) with hay fed separately. Sheep 8, 8N, 186, 97 and 6 received this ration except when recorded as 'fed oil', which meant that 40 g. linseed oil/day was added to the ration, or 'fed glycerol', which meant that 20 g. glycerol/day was added. Sheep 44 always had the basal diet + glycerol. Sheep 9 received a diet of hay and grass cubes only. Samples were taken via rumen cannulas about 3 hr. after feeding.

RESULTS

Glycerol-fermenting bacteria

Growth in a medium without rumen fluid. Rumen contents from sheep 6 were diluted in medium 1 and incubated for 3 days. Cultures which showed acid formation (up to $1/10^6$ dilution) were subcultured on solid medium 2 and incubated for 2 days. Acid-producing colonies were transferred to a liquid medium (medium 1

without agar). A mixed flora grew in the initial cultures, but the chief bacteria which grew on subculture were large, thick Gram-positive rods from dilutions up to $1/10^4$. From the initial $1/10^5$ and $1/10^6$ dilutions Gram-positive cocci were obtained which gave a weak acid reaction with glycerol; this activity was lost on subculture. The Gram-positive rods were 2μ to $5\mu \times 1\mu$, did not grow aerobically, on blood agar they gave β -haemolysis. Colonies were 2.5 mm. in diameter, smooth, whitish, translucent with slightly raised centre and uneven edge. Glycerol was fermented to give acid, and glucose, maltose, lactose and sucrose gave acid and gas; mannitol and salicin were not fermented. In litmus milk a stormy clot reaction was given and the lecithinase test was positive within 48 hr. The organisms were identified as *Clostridium perfringens* (Welchii).

Growth in a medium with rumen fluid incubated under hydrogen. Tenfold dilutions of rumen contents from sheep 6 (fed glycerol) were prepared in sterile saline, and 1 ml. portions of the dilutions mixed with medium 3 in Petri dishes and incubated under hydrogen for 3 days. Acid-forming colonies appeared up to $1/10^3$ dilution. The primary colonies were subcultured into medium 3 without agar, and further subcultures made from those cultures which showed acid formation. From the initial $1/10^3$ dilution some very small Gram-positive rods were obtained which did not survive further subculture. From the $1/10^2$ dilution Gram-negative rods of different sizes were obtained; they were non-motile and straight or slightly curved with some variation in length. Organisms grown aerobically on medium 3 had large capsules. Anaerobically very small capsules were formed and the organisms tended to be short and coccoid. Grown anaerobically and aerobically these bacteria were non-haemolytic. Colonies growing aerobically at 38° on medium 3 containing different amounts of rumen fluid were always opaque, mucoid, raised with entire edge, creamy white and about 3.5 mm. diameter; anaerobically the colonies were 0.5–1.75 mm. diameter and not mucoid. These bacteria fermented dulcitol, inositol, glycerol, mannitol, sorbitol, xylose, arabinose, rhamnose, aesculin, salicin, glucose, galactose, lactose, trehalose, dextrin and starch, giving acid and gas; adonitol and inulin were not fermented. Litmus milk after 17 hr. gave acid without a clot; at 48 hr. a soft clot had formed. Catalase reaction, nitrate reduction, Voges-Proskauer reaction and growth in Koser's citrate medium were all positive. Indole, methyl red and Eijkman tests, gelatin liquefaction and examinations for motility were all negative. The bacteria were classified as *Aerobacter aerogenes*.

Growth in media with rumen fluid incubated under carbon dioxide. Cultures were made of dilutions of rumen contents from sheep 6 (fed glycerol), 8, 9 and 44. Medium 4 was used for all samples except that from sheep 8, in which case the medium base was modified by adding rumen fluid to only 20 % (v/v) and agar to 2 % (w/v) and omitting the indicator. This modified medium 4 was used without glycerol or acetate, or with glycerol alone, acetate alone, or glycerol + acetate in the concentrations given under Methods. These media were inoculated with dilutions of rumen fluid (from sheep 8) prepared in a fluid similar to that of Doetsch, Robinson & Shaw (1952). All four media gave from sheep 8 a growth consisting predominantly of curved Gram-negative rods up to $1/10^9$ dilution. Growth was denser in the media with added glycerol. From the medium + glycerol alone isolations were made of a curved Gram-negative rod (A/10/B), and a very small Gram-negative coccus, the latter not surviving continued subculture.

Cultures from sheep 44 showed a growth predominantly consisting of curved Gram-negative rods up to $1/10^{12}$ dilution. No isolations were made from this experiment.

Cultures from sheep 6 (fed glycerol) made in medium 4 gave good growth and acid formation up to $1/10^8$ dilution; again Gram-negative rods predominated. From the $1/10^7$ and $1/10^8$ dilutions subcultures were made into medium 5. The pure cultures finally obtained and examined in detail were all from the $1/10^8$ dilution. There were six isolates (numbered, 3, 6, 7, 8, 9, 12) all strictly anaerobic Gram-negative curved rods with pointed ends, motile, evenly stained in young, vigorously growing cultures, but in older cultures showing granulation and uneven staining. The rods were $1.8\mu \times 0.4\mu$ to $3\mu \times 0.6\mu$, without capsules or iodine-staining polysaccharide. Growth on glycerol slopes (medium 5) gave opaque, light-brown colonies with a creamy periphery. Deep colonies were similar, but the centres were very brown and this colour deepened on prolonged incubation. Suspensions of organisms in saline were sometimes very dark-coloured, almost black.

Table 1. *Reactions of glycerol-fermenting selenomonads*

Substrate	Isolation number and reaction										
	3	6	7	8	9	12	17	18	19	20	21
Xylose	—	—	—	—	—	—	+	+	+	+	+
Starch	+	+	+	+	+	+	—	—	—	—	—
Dextrin	+	+	+	+	+	+	—	—	—	—	—
Sorbitol	+	+	+	+	+	+	—	—	—	—	—
Dulcitol	—	—	+	—	—	—	—	—	—	—	—
Inulin	—	—	—	—	—	—	+	+	+	+	+
Melezitose	+	+	+	+	+	+	—	—	—	—	—
Amylose	+	+	+	+	+	+	—	—	—	—	—
Nitrate reduction	+	+	+	+	+	+	—	—	n.t.	—	—

* Inulin fermentation much slower than other reactions.

n.t. = not tested.

All isolates fermented cellobiose, maltose, glucose, sucrose, glycerol, lactose, mannitol, galactose, arabinose, raffinose, salicin, aesculin, melibiose, ribose, fructose, sodium lactate, mannose, glucose-1-phosphate, glucosamine HCl. No isolates fermented trehalose, rhamnose, inositol, adonitol, xylan, cellulose, glycogen, sorbose, sodium acetate, fumarate, citrate, pyruvate, succinate. No isolates liquefied gelatin, formed indole, acetyl methyl-carbinol, or catalase, or grew aerobically. All formed H_2S and were motile. All grew at 30° and 38° , but not at 18° or 50° .

Cultures were also isolated from sheep 9 in the same manner. In the initial cultures growth and acid formation took place up to $1/10^9$ dilution. Pure cultures were obtained from this dilution (isolates labelled 17, 18, 19, 20, 21). In morphology and growth the cultures were similar to those from sheep 6 being motile curved Gram-negative rods $1.6\mu \times 0.6\mu$ to $3\mu \times 0.6\mu$. The biochemical reactions of all cultures are given in Table 1. Six isolates tested (numbers 3, 6, 9, 12, 17, 21) showed no lipolytic activity, and two isolates (3, 17) tested did not hydrolyse tributyrin. All isolates grew in medium 4 with 10% or 20% (v/v) rumen fluid or in a casitone + yeast medium without rumen fluid, and growth in a rumen fluid-type medium (similar to 4) was much better when casitone or yeast extract was added. As shown later, acetate was not utilized along with the glycerol, but it appeared to act as a growth factor, as growth in medium 4 + acetate was much better than without acetate at 24 hr.; after 48 hr., however, growth was much the same in each medium.

These curved Gram-negative glycerol-fermenting rods appear on morphological grounds (see Fig. 1) to belong to the genus *Selenomonas*. Since they all ferment lactate they may be classified as *S. ruminantium* var. *lactilyticas* (Bryant, 1956).

Lipolytic bacteria

Growth in saliva-based media. Experiments were made in which dilutions of rumen contents were inoculated into medium 10. Although growth was obtained, little lipolysis appeared to be taking place; the following results are typical. Tenfold dilutions of rumen contents from sheep 8N (fed linseed oil) were made in the diluting fluid and portions transferred to tubes of medium 10, medium 10 without glucose, and medium 10 without glucose and yeast extract. The cultures were incubated vertically in tubes in a shaker with a short, rapid travel. The shaker was kept going for about 7 hr. out of every 24 hr. during an 8-day period of incubation. The linseed oil emulsion made all the tubes too optically dense to see whether growth had taken



Fig. 1. Arrangement of flagella on glycerol-fermenting selenomonas. Not to scale.

place, but examination of stained films showed that a mixed growth of bacteria with many Gram-positive cocci occurred in medium 10 tubes up to $1/10^6$ dilution. From $1/10^7$ to $1/10^{12}$ dilutions the growth was mainly small indeterminate Gram-negative rods. Tubes of medium 10 minus glucose showed a similar growth, but the Gram-positive cocci were slightly more numerous in the greater dilutions; Gram-negative rods were always present. The tubes of medium 10 without glucose and yeast extract showed a few bacteria in dilutions $1/10^1$ and $1/10^2$, but nothing at greater dilutions. A representative number of tubes were taken for determination of lipolysis; the results are shown in Fig. 2. It will be seen that although there was some extra acid formed in cultures in medium 10, with and without glucose, the extent of lipolysis was similar to that produced by dilution of the rumen fluid in medium 10 without glucose and yeast extract, and no lipolysis due to growth of the bacteria in the greater dilutions was detected.

Growth in rumen-fluid based media. Preliminary tests established the composition of the medium which would give a stable emulsion of linseed oil and allow growth of bacteria. The following are the results of a preliminary experiment with medium 11. Dilutions of rumen fluid from sheep 8N in medium 11 were incubated in a nearly horizontal position on a rocking shaker making 1 rock per minute, as it was thought

that the shaker previously used was too violent. This slow rocking kept the linseed oil emulsion fairly well dispersed, only a slight amount of oil appearing on the surface of the medium. Incubation was continued for 5 days. The 5- to 6-day incubation period was initially used because it appeared to give greatest growth on visual examination of cultures; it was later confirmed that with pure cultures the viable count was higher after incubation for 6 days than for 3 or 10 days. Examination of cultures showed a mixed growth, with many Gram-positive cocci and Gram-negative rods of different shapes and sizes, up to $1/10^9$ dilution, the greatest made, except for dilutions $1/10^7$ and $1/10^8$ where the Gram-negative rods were present in very small numbers. The extent of lipolysis was determined on all tubes and is shown in Fig. 2. The result for the $1/10^5$ dilution may be low because of a mishap in the extraction, but it is evident that more lipolysis was taking place in general in the greater dilutions than could be accounted for by dilution of the rumen fluid; it appeared to be connected with the presence of Gram-negative rods, so more trials of the medium

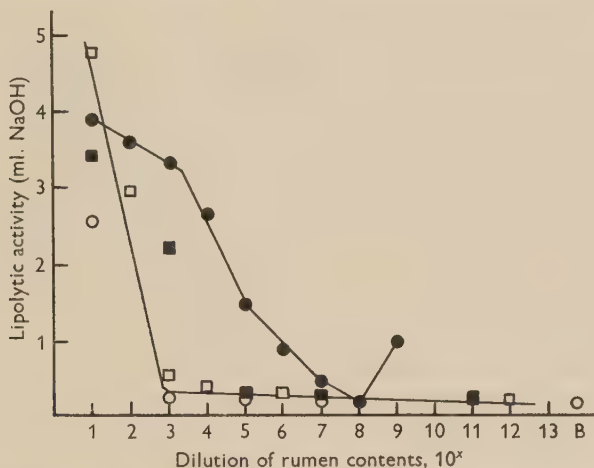


Fig. 2. Lipolytic activity of dilutions of rumen contents cultured in different media. Saliva-based medium: with linseed oil + glucose, □; linseed oil alone, ■; linseed oil without nitrogen source, no bacterial growth, ○. Rumen fluid-based medium: linseed oil + glucose, ●.

were made. For the isolation of lipolytic bacteria dilutions of rumen contents from sheep 8N (fed linseed oil) were made in medium 11 and incubated for 5 days. Stained films showed a mixed flora including Gram-positive cocci up to the $1/10^6$ dilution; from the $1/10^6$ to $1/10^9$ dilutions Gram-negative rods of different morphology were the organisms observed. All the dilutions except $1/10^9$ showed lipolysis and from the original $1/10^7$ and $1/10^8$ dilutions three isolates of lipolytic bacteria were obtained by dilution and subculture on alternate liquid and solid media (12 and 13). These were small Gram-negative rods labelled 7ES, 8ES and 8ER. The lipolytic activity in liquid medium (12) was tested at intervals while the cultures were kept; it did not diminish on subcultivation. Up to about 70 % hydrolysis of the oil occurred in 6 days. In morphology, growth characteristics and biochemical reactions all these isolates were similar, except that isolate 7ES grew much more poorly than the others and did not keep so well in stock cultures. The organisms were strictly anaerobic,

Gram-negative rods variable in size, but generally 1.2μ to $1.8\mu \times 0.4\mu$ in young cultures. They were more or less curved depending on the size of the given organism. Organisms in older cultures became faintly staining and developed a central granule; these organisms were generally larger (up to 3μ to $3.6\mu \times 0.4\mu$). Later the organisms seemed to disintegrate leaving only a granular mass. The organisms were actively motile with a single polar flagellum. No iodine-staining polysaccharide or definite capsules were seen, but organisms of isolates 8ES and 8ER were buried in masses of slime. Isolate 7ES was not so noticeably slimy. In glycerol agar colonies were lenticular and brownish. Towards and on the agar surface, colonies were round, raised, whitish and mucoid; on continued incubation, the colonies spread to give a very slimy growth all over the agar surface. In linseed oil agar roll tubes colonies were minute or invisible to the naked eye unless incubation was continued for a long time, and growth was usually noticed only by the circular clear areas which developed in the agar. Clear zones, taken to indicate esterase activity, were formed in the tributyrin medium.

These bacteria hydrolysed linseed oil, but did not apparently utilize the resultant long-chain fatty acids and no growth occurred in a medium containing the acids from hydrolysed linseed oil. Fermentation tests were originally carried out in medium 12 + the appropriate sugar in place of oil. Of the substances listed in Table 1 only glycerol, fructose and ribose were fermented, the first more quickly than the others. However, in all cases growth was not luxuriant, so some of the tests were repeated using medium 14. Growth was much better in this medium, but only the same substances were utilized. In medium 12 with glycerol alone the final pH value was 5.6, in medium 14 it was pH 5.0. Hydrogen sulphide was formed, nitrate was not reduced to nitrite, indole was not formed, nor was gelatin hydrolysed. Growth occurred at 38° , but not at 20° , 30° or 50° . In rumen fluid-based media growth was somewhat better when 30 % or 40 % rather than 10 % or 20 % (v/v) rumen fluid was used. These lipolytic bacteria could not be identified with any of the rumen bacteria so far described.

Products of glycerol fermentation

Some of the bacterial isolates were tested for the products of glycerol fermentation. The mixed flora growing from $1/10^3$ and $1/10^4$ dilutions of rumen contents inoculated into medium 1 produced formic, acetic, propionic, butyric, isovaleric and caproic acids and four clostridial isolates from this medium all produced formic, acetic and butyric acids. Other products were not tested for. The Gram-negative curved rods were all very similar in fermentation products. Isolate A/10/B, isolated from sheep 8 in medium 4 containing only glycerol (see above), produced mainly propionic acid when grown with glycerol or glycerol + acetate. The six isolates of the selenomonads from sheep 6 all produced mainly propionic acid in a glycerol + acetate medium (6). Selenomonad isolate 3 produced in one experiment (μ mole acid/ml. medium): propionic, 50.4; lactic, 8.2; succinic, 14.6; acetic, 4.2; no formic or butyric acids. These products accounted for 111.3 % of the carbon of glycerol utilized. In other experiments very slight growth appeared to take place in the medium without glycerol with the formation of some volatile fatty acids. This might account for this high carbon recovery. Selenomonad isolate 21 in the same medium

produced mainly propionic acid, with some lactic acid (succinic acid not tested for, no carbon balance done). The lipolytic bacteria which utilized glycerol in medium 14 without oil produced mainly propionic acid. In one experiment the products from isolate 7ES were (μ mole acid/ml. medium): acetic, 3.4; propionic, 36.2; butyric, 0.6; succinic, 11.0; no formic or lactic acid. These products accounted for 89.1 % of the carbon of the glycerol fermented. Isolates 8ES and 8ER behaved similarly. Isolate 8ES produced in one experiment (μ mole acid/ml. medium): acetic, 6.0; propionic, 54.0; butyric, 1.0; succinic 8.0; no formic or lactic acid; carbon recovery 90.0 %. There was no obvious gas formation with glycerol media, but since no quantitative measurements were made, some CO₂ or bacterial slime might account, partially at least, for the low carbon recoveries.

Ammonia utilization or formation

The utilization of ammonia by nine isolates of selenomonads and the three lipolytic isolates when grown in either of the media 8 and 9 was not definitely detected after growth for 2 days. Except in three cases the ammonia concentration in the growing culture was within 0.5 μ mole/ml. of that of the control. In the three cases the difference was about 1 μ mole/ml., but it was not consistent and was ignored. Some rumen bacteria have been shown to utilize ammonia when growing in media in which this compound was almost the only source of nitrogen, others have been found to need amino acids or peptides as well as ammonia. The two media described here should have provided both conditions. Growth in medium 9 with added yeast extract was much better than in medium 8.

Counts of lipolytic bacteria in rumen contents

Viable counts of lipolytic bacteria in the rumen contents of sheep 9, and of sheep 97 and 186 on the basal ration alone and fed linseed oil were made on medium 13 in roll tubes. The number of clear zones in the medium after incubation for 6–7 days was noted. As it was very difficult to see some of these zones, only approximate counts were made. Clear zones were found in media inoculated from sheep 9 up to 1/10⁹ dilution, from sheep 97 to 1/10¹⁰ and from sheep 186 to 1/10⁹ on each ration. Although colonies of bacteria not visibly utilizing the oil could be seen, in the smaller dilutions especially, the zones of clearing contained colonies that were barely visible to the naked eye, as with the pure cultures of lipolytic bacteria. On prolonged incubation the colonies and the clear zones became larger, but by this time the majority of bacteria appeared to have disintegrated (compare the pure cultures) and no definite morphological structures could be identified.

DISCUSSION

It is evident from the experiments with sheep 8 that the addition of glycerol to a rumen fluid medium did not lead to the growth only of bacteria capable of utilizing this compound; many bacteria seem to have requirements such that they can grow on the substrates provided by rumen fluid + yeast extract. However, the media used here should have permitted the growth of a good selection of bacteria able to utilize glycerol even if others grew also. The results show that whilst bacteria like

those found in other habitats play some part in the rumen fermentation of glycerol, they are present only in small numbers. The results are similar to other reports in which different media have shown similar numbers of bacteria such as *Aerobacter aerogenes* to be present in the rumen. The numbers in which the selenomonads grew show that they must form a major component of the glycerol-utilizing flora. It is also evident that they are a basic part of the rumen flora and are not found solely or even mainly in the rumen of sheep fed glycerol-containing diets. These selenomonads, however, can utilize a large selection of 'sugars' and so would be expected to exist on substrates provided by almost any diet. Although there are obvious differences in fermentation reactions between the isolates of selenomonads from sheep 6 and 9, in general the reactions agree with those of the two bovine strains of lactate-fermenting selenomonads described by Bryant (1956) which also showed some strain differences. Isolates 3 and 21 were also tested qualitatively for volatile fatty acids formed by fermentation of glucose; propionic acid and acetic acid were the only ones detected. In this these bacteria are similar to *Selenomonas ruminantium* var. *lactilyticas*, both strains of which produced mainly propionic and acetic acids from glucose. In general the size of the isolated selenomonads was less than that of the selenomonads seen in the rumen; further experiments are in progress to see whether the bacteria described here are really typical large rumen selenomonads which have become smaller on cultivation *in vitro*. In the absence of acetate, glycerol fermentation *in vitro* was much slower than the fermentation of many other carbohydrates, so that the presence of acetate in the rumen would aid in the rapid utilization of glycerol by the selenomonads. Whilst glycerol-fermenting bacteria other than the selenomonads and lipolytic rods are present in the rumen and would contribute small amounts of mixed volatile fatty acids to its contents (e.g. clostridia), the selenomonads and the lipolytic bacteria described here must constitute a significant part of the flora and their products of fermentation of glycerol should bear some relationship to those found in the rumen. The main fermentation product in all cases was propionic acid, with some lactic and succinic acid. The amounts of volatile fatty acid formed differed in different experiments, but were always less than the theoretical amounts provided by the complete conversion of the glycerol utilized; in some cases the amount was only 50–60 % of the theoretical. These results are similar to those found with whole rumen contents *in vitro* where only a fraction of the glycerol fermented could be accounted for as volatile acids, considered as propionic (cf. Garton *et al.* 1961). If the other products were lactic or succinic acids one might expect them to be rapidly fermented by the whole rumen contents giving, again, propionic acid. Thus, either other so far unidentified products are formed, or the fermentation of lactate and succinate takes place so slowly under the *in vitro* conditions used that these products accumulate. That this could happen is shown by the fact that small amounts of lactic acid were found amongst the products of glycerol fermentation by the selenomonads, although these bacteria will utilize lactate when it is the sole substrate present. The selenomonads and the lipolytic bacteria did not utilize ammonia or produce it under the conditions of the test, so no obvious reason appears for the presence of glycerol having an effect on ammonia levels in the rumen.

Other studies on lipid metabolism by bacteria have been mainly confined to aerobic or facultatively anaerobic bacteria growing on substrates containing glycerides of lower fatty acids. In some cases the hydrolysis of esters of the higher fatty

acids has been demonstrated, again principally with aerobes or facultative anaerobes. In earlier (unpublished) experiments on rumen contents the presence was shown of a small number (about 10^4 /ml.) of bacteria of the genus *Bacillus*, which were capable of growing on relatively simple media and of hydrolysing tributyrin. The lipolytic bacteria described here seem to be the only ones so far isolated from the rumen which will hydrolyse triglycerides of long-chain fatty acids. Although morphologically similar to many types of rumen bacteria they differ from all known species in their limited fermentation reactions. It would thus appear that they might be put in a new classification, but it is probably better to leave this until further strains are isolated. These bacteria are probably not the only ones which will hydrolyse glycerides in the rumen, but from the numbers present (about 10^8 /ml.) they would appear to have a large part in this action. Again, the bacteria seem to be regular inhabitants of the rumen, and not to be encouraged by feeding linseed oil or glycerol, but all the rations fed to the animals would contain some, if only a very small amount, of lipid. Although these bacteria readily hydrolyse linseed oil they do not apparently metabolize the higher fatty acids formed; this is in accordance with the experiments in which rumen contents were incubated with triglycerides (Garton *et al.* 1961) where the liberated acids accumulated quantitatively. Further experiments will be needed to see whether these lipolytic bacteria are also responsible for the hydrogenation of unsaturated fatty acids in the rumen. Although the lipolytic bacteria described here were tested for hydrolytic activity only towards linseed oil and tributyrin, it is possible that they will also hydrolyse other triglycerides, as do mixed rumen bacteria (Garton *et al.* 1961).

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The Isolation and Characterization of Bacteriophages from *Listeria monocytogenes*

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SUMMARY

The bacteriophages of *Listeria monocytogenes* have been studied with respect to isolation techniques and their use as diagnostic tools and as aids in epidemiological investigations. The occurrence of lysogeny was investigated in 123 strains isolated from human and animal sources throughout the world. Conventional procedures for isolation of phage were unreliable with *Listeria* since lysogenic strains did not always, by spontaneous lysis, release a detectable amount of phage. However, after exposure to ultraviolet radiation, such strains were induced to produce up to 10^7 plaque-forming particles/ml. Some strains which did not release phage produced substances after irradiation possibly analogous to colicines. The lytic spectrum of 11 phages against 149 strains of *Listeria* was studied and a system of classification, with five of these phages, was used to place 127 of these strains in 8 phage types. Nearly all of the untypable strains were rough, undergoing dissociation, or were lysogenic. Phage susceptibility appeared to be closely associated with the serological type of the strain, but showed no relation to the animal source or the geographical origin. These studies indicated that *Listeria* phages could be used as a means of generic identification and also as a substitute for or an adjunct to serological typing in epidemiological investigations.

INTRODUCTION

The increasing frequency of reports of human and animal listeriosis tend to give this disease a measure of importance not formerly accorded it (Seeliger, 1958; Gray, 1959; Welshimer & Winglewish, 1959). However, the epidemiological picture of listeriosis is incomplete, and neither biochemical nor serological procedures have been completely satisfactory in clarifying this picture. The successful application of bacteriophage typing to other bacterial genera suggests that such a method might be of value in tracing outbreaks of listeriosis or in proving transmission of the organism. Moreover, identification and differentiation of *Listeria* from other genera of Gram-positive rods still presents a problem in many laboratories. All too frequently, cultures of *Listeria* are erroneously identified as diphtheroids. Serological procedures for the identification of *Listeria* are expensive and are not readily adapted to most small laboratories. Bacteriophage filtrates, on the other hand, are

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inexpensive, easily prepared, relatively stable, and can be used by many laboratories not able to maintain a complete stock of reagents and typing sera for diagnosis of the less frequently encountered bacterial pathogens. Therefore, an evaluation of specific phage as an aid to the identification of genus *Listeria* seemed warranted.

Bacteriophage typing was first established as a practical and precise means of demonstrating subtle strain differences by Craigie & Yen (1938) in their classic studies on *Salmonella typhosa*. The use of adapted phage permitted them to detect differences among strains of Vi-positive *S. typhosa* which were undetectable by biochemical and serological means. This method found wide acceptance in tracing typhoid outbreaks and has since been applied to other groups of bacteria including *S. paratyphi B* (Felix & Callow, 1951), *Shigella sonnei* (Hammarström, 1949), and *Pseudomonas pyocyanea* (Warner, 1950). In addition, sensitivity to phage has been found to be a reliable method of identifying members of the genus *Salmonella* (Cherry, Davis, Edwards & Hogan, 1954; Chi, 1956; Pickett & Laughner, 1960), *Malleomyces* (Smith & Cherry, 1957), and for differentiating *Pasteurella pestis* from *P. pseudotuberculosis* (Gunnison, Larson & Lazarus, 1951). Phage typing of *Staphylococcus aureus*, originally developed by Fisk (1942*a, b*), and later modified by others (Wilson & Atkinson, 1945; Williams & Rippon, 1952; Hood, 1953; Blair & Carr, 1953, 1960), is now extensively used in epidemiological investigations. *Listeria monocytogenes* phage was described by Schultz (1945) but, except for a preliminary report (Sword & Pickett, 1958), phage typing of this species has not previously been reported.

To establish the reliability of a phage typing scheme, we first evaluated both the extra- and intragenetic specificity of *Listeria* phages. The former was pertinent in view of reported serological relationships between *Listeria* and both *Staphylococcus aureus* (Drew, 1946; Seeliger & Sulzbacher, 1956) and enterococci (Seeliger, 1955*a*). Also, phage pools were examined for evidence of interference among heterologous phages; the activity of undiluted stock phages and preparations diluted to routine test doses (RTD; as defined by Williams & Rippon, 1952) were compared; and the relationship between colonial dissociation of the host and its susceptibility to phage was studied.

METHODS

Test strains and their routine culture. The 149 strains of *Listeria* used in this work were from human and animal sources throughout the world. They were obtained from at least twelve animal species through the courtesy of workers in ten different countries. The identity of all strains was confirmed by observation of motility in semi-solid agar as well as microscopic and colonial morphology. All cultures were maintained on Tryptose agar (Difco), stored at 4°, and transferred at 6-month intervals. Blood agar base (Difco) and heart infusion broth (Difco) were used for propagating phages and for typing procedures.

Serological properties. In many cases information about the serotype was supplied by the source laboratory. However, to confirm this and to identify untyped cultures the procedures for immunization, agglutinin-absorption, and typing as described by Paterson (1939, 1940) and Seeliger (1955*b*) were used. The following strains from the National Collection of Type Cultures (London, England) were used as antigens: 2167, 5105, 5214 and 5348.

Isolation of bacteriophage. Cultures of certain strains (44-24L, 44-50, 44-51, and 44-67L) which have been shown by the method of Fisk (1942*a, b*) to carry phage, did not always contain enough free phage particles to be readily detectable when spotted on indicator strains. Preliminary use of the ultraviolet induction technique of other workers (Liegeois-Muller & Fredericq, 1952*a, b*; Thibaut & Fredericq, 1952) yielded promising results with these strains. A mineralight lamp (Model V 41 Ultraviolet Products, Inc. San Gabriel, Calif., U.S.A.) which emitted approximately 52 % ultraviolet radiation with a wavelength of 2537 Å was used. The lamp (with filter) at a distance of 45 cm. from the object being irradiated was shown by a General Electric germicidal ultraviolet intensity meter to produce 685 ergs/cm²/sec. Irradiation for 1-2 min. at a distance of 25 cm., followed by incubation at 37° for 90 min. yielded maximal phage titres, often as high as 10⁷ plaque-forming particles/ml. *Listeria* strains to be irradiated were grown overnight at 37°. Small volumes (2-3 ml.) were spread in a thin layer over the surface of open Petri dishes and irradiated. The dishes were swirled periodically during irradiation to provide maximum exposure, and the procedure was carried out in semi-darkness to avoid photo-reactivation. After irradiation and incubation the strains were spotted at spaced intervals by Pasteur pipettes on plates seeded with indicator strains. The indicator strains were included in a thin layer of semi-solid agar on the surface of blood agar base plates as described by Adams (1950). The spots were allowed to dry and the plates incubated at 37°. In this manner, each strain could serve in turn as the substrate on which the others were spotted and all desired combinations of strains could be achieved. A group of 123 strains was tested for the presence of phage by spotting on 52 strains chosen to include those of as many geographic, host and serological groups as possible.

Purification and preparation of stock phage suspensions. Spotted areas which showed the presence of plaques or confluent lysis were selected for further study. A small amount of the semisolid agar from such areas was removed by a wire loop and shaken in 1 ml. broth to elute the phage; 0.1 ml. of this broth was then incorporated into semi-solid agar overlays along with the appropriate seed organism. In this manner newly isolated phages were transferred two to three times from single plaques to insure purity of the final phage preparation. Stock phage preparations were obtained by harvesting from agar overlay plates by a modification of the method of Swanstrom & Adams (1951). Phage suspension was incorporated into seeded agar overlay on blood agar base plates. After incubation the agar overlay was scraped from the surface of the agar base and suspended in heart infusion broth. Phage was recovered from the agar by elution overnight at 4° followed by centrifugation at 2000 *g* for 30 min. The resultant supernatant fluid was sterilized by adding 0.001 % (w/v) thymol or by passage through a millipore HA filter pad (Millipore Filter Corp., Bedford, Mass., U.S.A.) and stored at 4°. Titres of phage suspensions ranged from 10⁸ to 10¹¹ plaque-forming particles/ml. Preparations were stable at 4° for at least 6 months and often for as long as 1 year.

Electron microscopy. Phage particles were concentrated from stock preparations by centrifugation at 15,000 *g* for 1 hr. in a Spinco (Model L) ultracentrifuge. The deposits were resuspended to one-tenth the original volume with broth and centrifuged at 2000 *g* for 1 hr. to remove large particles. High speed centrifugation was then repeated on the supernatant fluid, and the final pellet suspended in 1 ml. of

neutral isotonic ammonium benzoate (1.8 %, w/v). This suspension was spotted on electron microscope specimen screens covered by a collodion membrane (Wyckoff, 1949). All screens were air dried, shadowed with palladium and examined with an RCA EMU electron microscope. Four phages (82, 135, 51L, 137) were examined by this method.

Routine procedure for phage typing. Agar overlay plates seeded with the bacterial host were prepared as described and spotted with phage suspensions by means of capillary pipettes. As many as twenty spots could be applied but ordinarily only a set of 4 phage preparations was used (a pool of phages 83, 135, 51L, 24L, 87). Since comparison of the lytic spectra of undiluted phage and phage diluted to the routine test dose revealed no differences, the former was used for typing.

RESULTS

Isolation of bacteriophage

Forty-three of the irradiated and spotted cultures lysed one or more of the 52 indicator strains of *Listeria*. Purified phage suspensions were obtained from 12 of these 43 by transfer from single plaques. No phage was isolated from strains of serotype 3 even after cross-testing a group of 10 such strains.

Demonstration of bacteriocines after irradiation

Thirty-one of the 43 previously mentioned lytic combinations failed to yield single plaques or phage when tested further. However, the lytic or inhibitory effect had a definite host range against the indicators. This effect was dependent on ultra-violet irradiation; unirradiated cultures did not cause lysis or inhibition of growth when spotted on indicators. This phenomenon was not further examined, but appeared to be referable to colicine-like agents. Such substances have been found in several bacterial species other than *Escherichia coli*, but have not been reported previously from *Listeria monocytogenes*. Jacob, Lwoff, Siminovitch & Wollman (1953) suggested the term 'bacteriocine' for substances possessing the general properties of colicines.

Electron microscopy

Plate 1, fig. 1, shows the morphology of phage 83. No morphological differences were found among the 4 phages examined. The phage heads appear to be about 85–90 m in diameter while the tails measure $265 \times 15 \text{ m}\mu$. A terminal knob on the tail (not shown here) was present in some preparations, but was never as pronounced as with some phages.

Effect of Listeria phages on heterologous species

To establish generic specificity undiluted stock phages 83, 135, 51L, 24L and 87 were spotted on the following heterologous bacterial species: *Corynebacterium pyogenes*, *C. pseudodiphtheriticum*, *C. pseudotuberculosis*, *Erysipelothrix rhusiopathiae*, *Lactobacillus arabinosus*, *L. brevis*, *L. buchneri*, *L. casei*, *L. fermenti*, *L. lycopersici*, *Staphylococcus aureus*, *Streptococcus equi*, *S. faecalis*. In no instance was lysis observed.

Lytic spectrum of phages against strains of Listeria

Eleven phage preparations (phages 23, 24L, 51L, 61, 71, 83, 87, 113, 132, 135, 137) were spotted on 149 strains of *Listeria*. The lytic spectrum of each preparation was usually restricted to strains of the serotype from which that phage had been obtained. Furthermore, there appeared to be two rather broad but distinct groups of strains with respect to phage susceptibility, namely, those lysed by one or more of the following phages: 23, 24L, 71, 83, 132, 135 and 137, and those lysed by one or more of phages 51L, 61 and 113. Since several of these phages possessed almost identical patterns of activity, only those with distinctly different spectra were used in developing a phage typing scheme. For this, a system of classification using only phages 83, 135, 51L, 24L and 87 was devised (Table 1). Phages 83 and 135 were quite similar, having only minor differences in their ranges of activity. Therefore these two were mixed into a single pool. The spectrum of phage 51L appeared to complement that of the pool (83+135). By spotting this battery of four phage preparations on an unknown strain, the latter could be classified in one of the eight groups listed in Table 1 or else was insensitive to all five phages. Only 22 out of 149 strains (14.7 %) were not sensitive to bacteriophage. Of these untypable strains, 2 were lysogenic and hence immune to phages related to their lysogenizing phage, and 19 were, colonially, non-smooth or frankly rough.

Table 1. *Bacteriophage sensitivity patterns of Listeria strains*

Phage type	Reactions with typing phages			
	83/135	51L	24L	87
1	+	+	+	.
2	+	+	-	.
3	+	-	+	.
4	+	-	-	.
5	-	+	+	.
6	-	+	-	.
7	-	-	+	.
8	-	-	-	+
Untypable	-	-	-	-

+ = lysis; - = no reaction.

Polyvalent pooled phage suspensions for generic identification of Listeria

A polyvalent pool of phages 83, 135 and 51L was spotted on the group of 149 strains mentioned above. The results obtained with the pool expressed the cumulative effect of the individual phage spectra on all strains tested and suggested that the phages could be usefully employed for identification of the genus *Listeria*.

Host and geographic distribution of phage types

An attempt was made to determine whether phage sensitivity showed any relation to the animal host or to the geographic origin of the strain (Tables 2, 3). None was found. Thus it appears that phage type, like the serological types described by Paterson (1939, 1940), is independent of source. However, certain patterns were noticeable within limited groups of strains. We found that two strains (44-17, 44-18)

isolated by Line & Cherry (1952) from infants in the same hospital ward had identical patterns of phage sensitivity. These workers suggested transmission from one mother to the other since the mothers shared a bathroom. We also found that 5 out of 6 strains isolated by Roine, Raitio & Vartiovaara (1953) in Finland from an outbreak of listeriosis in guinea pigs showed similar patterns of phage sensitivity. Similar tendencies were noted with other strains. It may then be possible to use this phage typing system for epidemiological purposes.

Table 2. *Bacteriophage type distribution of Listeria strains according to animal source*

Phage type	Strains		Animal source						
	No.	%	Human	Bovine	Ovine	Avian	Rodent	Porcine	Unknown
1	3	2.0	1	.	2
2	1	0.7	.	.	.	1	.	.	.
3	49	32.2	6	7	9	9	8	2	8
4	6	4.0	3	.	1	.	2	.	.
5	5	3.3	.	3	2
6	53	35.5	8	10	22	4	6	1	2
7	5	3.3	2	1	.	.	1	1	.
8	5	3.3	5
Untypable	22	14.7	10	3	6	1	.	1	1

Table 3. *Bacteriophage type distribution of Listeria strains according to geographic source*

Phage type	Geographic source			
	N. America	Europe	Asia	Australia
1	2	.	1	.
2	.	1	.	.
3	28	20	1	.
4	1	5	.	.
5	1	3	1	.
6	36	12	5	.
7	2	2	.	1
8	2	3	.	.
Untypable	7	14	1	.

Relationship of phage type to serological type

The phage type of 51 serologically typed strains was determined. The group of strains was selected to contain representative numbers of all the serotypes. There was almost perfect correlation between serotypes 1 and 2 and sensitivity to the pool of phages 83 + 135. Serologically, only the flagellar antigen 'D' separates these two serotypes, and strains of serotype 2 are very rare. Strains of both serotypes 1 and 2 belong to phage types 3 and 4. Most strains of serotype 3 are phage-resistant but those which are sensitive are lysed only by phage 87 and therefore belong to phage type 8. The phage-resistant serotype 3 strains are all intermediate or rough colonial types. Nearly all strains of serotype 4 are lysed by phage 51L and therefore belong to phage types 5 and 6. Only one case of cross-activity between the pool of phages 83 + 135 and phage 51L was noted in this group. Hence it appears that there is good correlation between phage sensitivity and serological type.

DISCUSSION

The ultraviolet irradiation procedure used in this study was effective in increasing the phage titre of susceptible lysogenic cultures. Carrier strains (Lwoff, 1953) and non-inducible lysogenic cultures should be unaffected by such a procedure. However, if these strains did produce phage it would be as readily detectable by this procedure as with conventional cross-testing procedures. All phages isolated in this study were from lysogenic strains since all responded to ultraviolet induction. Thus, all are 'temperate' rather than 'virulent' according to Lwoff's definition (Lwoff, 1953).

Examination of those strains which were not sensitive to the lytic action of phage disclosed that most of these were not smooth, frankly rough, or lysogenic. These differences in susceptibility suggest that if only freshly isolated smooth strains were used, the finding of apparently untypable strains should be considerably diminished.

The failure of these *Listeria* phages to lyse strains of bacteria of other genera confirms their generic specificity and indicates that they could, in a polyvalent pool, safely be used as a means of positive generic identification of fresh isolates. The high rate of host susceptibility and phage specificity, as well as the relative ease and economy of procedure as compared with serological methods suggest that this group of phages can profitably be used either as a substitute for serological typing or as an adjunct to it in epidemiological investigations of listeriosis.

We wish to thank the many laboratories throughout the world who generously contributed cultures for this study. The authors are also indebted to Mr H. Froula and Mr E. Boller, Department of Engineering, University of California, Los Angeles, U.S.A., for their generous aid with the electron micrographs.

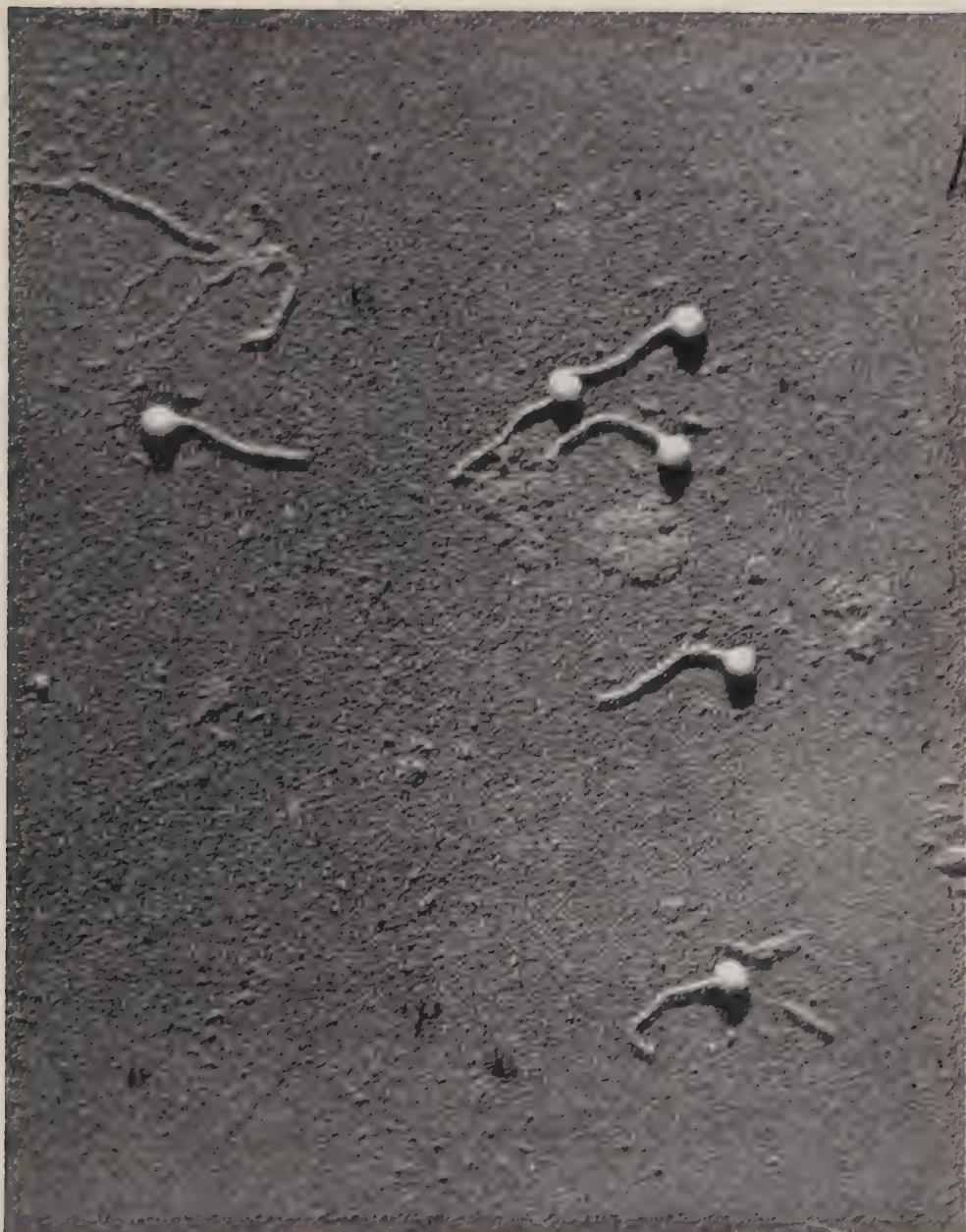
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EXPLANATION OF PLATE

Fig. 1. Electron micrograph of 83: $\times 65,000$.



The Nutrition of a *Lactobacillus acidophilus* Variant Isolated from the Duodenum of a chick

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SUMMARY

Allen, Stephens, Jaffe & Wakelam (1959) isolated a lactobacillus which they found to require a growth factor present in malt distillers' dried solubles. We have identified the organism as belonging to the subgroup *Thermobacterium* Orla-Jensen. It was grown through many serial passages in a chemically defined medium containing thymidine, and had no residual indispensable requirement for unidentified nutrients.

INTRODUCTION

Malt distillers' dried solubles (MDDS), made from the liquor remaining in the whisky still after the spirit has been distilled, have been reported to contain unidentified factors which promote growth in chicks (Wakelam & Jaffe, 1959). The feeding of MDDS to chicks changed the composition of the bacterial flora of the duodenum and caused an increase in the numbers of an unidentified species of lactobacillus (Allen, Stephens, Jaffe & Wakelam, 1959). Allen *et al.* isolated this organism and found that it required a growth factor present in MDDS. Further tests with a variety of feed supplements showed that stimulation of chick growth was correlated with growth-promoting activity for the *Lactobacillus* sp. It seemed probable that the factor which stimulated growth in the chicks was the same as that required by the *Lactobacillus* sp. Through the kindness of Drs K. A. Allen and J. Stephens we have been able to make a further study of the nutrition of this organism.

METHODS

Organism. Cultures of the organism can be obtained from the National Collection of Dairy Organisms (NCDO) at the National Institute for Research in Dairying, Shinfield, Reading, as culture no. NCDO 1417. It will be referred to in this paper as strain 1417. We have found that it belongs to the subgroup *Thermobacterium* Orla-Jensen and has the cultural characteristics of *Lactobacillus acidophilus* (see Rogosa & Sharpe, 1959) except that it does not ferment trehalose.

Maintenance of organism. The organism was maintained by monthly transfer in the basal medium (Table 1) supplemented with 10 % (v/v) of an aqueous extract of MDDS. (To 50 g MDDS were added 400 ml. water and enough $N-H_3PO_4$ to bring to pH 6.5. The mixture was heated in flowing steam for 30 min., cooled and centrifuged. The supernatant fluid was diluted to 500 ml. with water.)

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Cultures were first grown for 18 hr. at 37° in the liquid medium and were then transferred as stabs to the same medium solidified with 1.5 % (w/v) agar. After incubation for 24 hr. these stab cultures were stored at 2°.

Preparation of inocula for tests. The cultures used to inoculate the tests were grown for 18 hr. at 37° in the basal medium supplemented with 10 % (v/v) of the MDDS extract. They were diluted 1/10 with sterile 0.9 % (w/v) NaCl solution and one drop added to each assay tube.

Basal medium. The composition of the basal medium is shown in Table 1. For some tests the medium was modified by using the following mixture of amino acids to replace the hydrolysed casein and Tryptone: L-leucine, L-isoleucine, L-valine, L-lysine, L-arginine, L-methionine, 500 mg. each; L-glutamic acid, L-asparagine, 1 g. each; L-alanine, glycine, L-serine, L-aspartic acid, L-tyrosine, L-proline, L-histidine, L-phenylalanine, L-threonine, L-tryptophan, 200 mg. each; in 200 ml. of 5 × single strength medium.

Table 1. *Basal medium. Composition to give 5 × the concentration of final medium*

Acid hydrolysed casein*	(g.)	5	Riboflavin	(mg.)	1
Charcoal-treated Tryptone†	(g.)	5	Thiamine	(mg.)	1
Glucose	(g.)	10	Nicotinic acid	(mg.)	1
KH ₂ PO ₄	(g.)	3	Calcium pantothenate	(mg.)	1
K ₂ HPO ₄	(g.)	3	Pyridoxal ethyl acetal hydrochloride	(mg.)	1
Diammonium citrate	(g.)	0.6	Biotin	(μg.)	10
Solution of mineral salts‡	(ml.)	10	Folic acid	(μg.)	1
Tween 80§	(ml.)	1	p-Aminobenzoic acid	(μg.)	1
Adenine	(mg.)	5	Cyanocobalamin	(μg.)	1
Guanine	(mg.)	5	Ascorbic acid	(g.)	0.5
Uracil	(mg.)	5	Cysteine	(mg.)	50
Xanthine	(mg.)	5	pH adjusted to 6.2, and water added to 200 ml.		

* Allen and Hanburys, Ltd. 'Vitamin free' grade.

† Prepared as follows: 5 g. Tryptone (Oxo Ltd.) were dissolved in 400 ml. water, and the solution adjusted to pH 4.0 with acetic acid. Charcoal (10 g. Sutcliffe and Speakman Ltd.; Grade 5) was added, and the whole stirred for 10 min. and then filtered. The filtrate was adjusted to pH 6.0 with 10N-KOH solution, and a further 5 g. charcoal added. After stirring for 5 min. the mixture was filtered, and to the filtrate were added: DL-tryptophan (1 g.), L-tyrosine (200 mg.), L-phenylalanine (200 mg.), L-proline (100 mg.) and L-histidine (100 mg.). Finally, water was added to 500 ml.

‡ Contains MgCl₂·6H₂O, 20 g.; CaCl₂, 5 g.; FeCl₃·6H₂O, 0.5 g.; ZnSO₄·7H₂O, 0.5 g.; MnSO₄·4H₂O, 0.25 g.; CoCl₂·6H₂O, 0.25 g.; CuSO₄·5H₂O, 0.25 g.; VSO₄, 0.25 g.; Na₂MoO₄, 0.25 g.; dissolved in 1 l. distilled water with addition of N-H₂SO₄ to clear.

§ Polyoxyethylene sorbitan mono-oleate.

Test procedure. The growth promoting activities of the test preparations were judged by comparison with the activity of a 1/5 dilution of the MDDS extract. This 'standard' and the 'test' solutions were added to series of tubes in amounts of 1, 2, 4 and 8 ml., together with 2 ml. portions of 5 × single strength basal medium and water to make final volumes of 10 ml. The tubes were then capped and autoclaved at 115° for 10 min. After cooling to room temperature the tubes were inoculated and incubated at 37° for 24 hr. Growth responses were measured turbidimetrically with a Lumetron model 400 A photometer (Photovolt Corporation, 95 Madison Avenue, New York, 16 N.Y., U.S.A.).

RESULTS

Several proprietary extracts of yeast proved about as active as MDDS in promoting the growth of strain 1417. Yeast nucleic acid (British Drug Houses Ltd., Poole, Dorset, England) and herring sperm deoxyribonucleic acid (L. Light and Co., Ltd., Poyle Trading Estate, Colnbrook, Buckinghamshire, England) showed relatively little activity (about 2 % that of MDDS) but depolymerization of these compounds with cobra venom caused a considerable increase in their activity to equal, or slightly exceeding, that of MDDS. Ribose and deoxyribose were inactive. Adenosine, adenylic acid, guanosine, guanylic acid, cytidine and uridine were

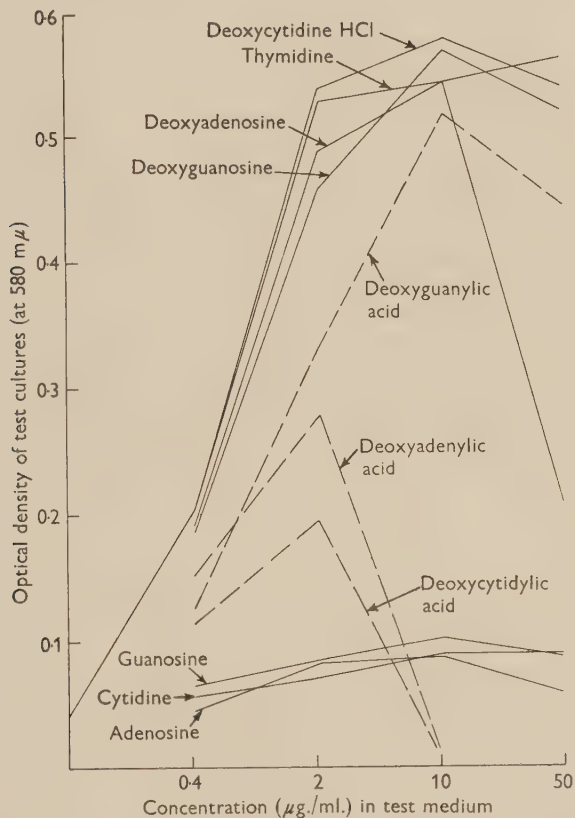


Fig. 1. Growth responses of strain 1417 to graded concentration of various nucleosides and nucleotides.

inactive or only very slightly active at 10 or 50 $\mu\text{g./ml.}$ But deoxyadenosine, deoxyguanosine, deoxycytidine and thymidine were highly active, giving at 2 $\mu\text{g./ml.}$ a growth response greater than that elicited by about 4 mg. MDDS/ml. Deoxyadenylic acid, deoxyguanylic acid and deoxycytidylic acid, at 0.4 and 2.0 $\mu\text{g./ml.}$, were somewhat less active than the corresponding deoxynucleosides. At 10 and 50 $\mu\text{g./ml.}$ deoxyadenylic acid and deoxycytidylic acid were inhibitory. The very slight activity of some of the ribosides tested might have been due to contamination

of these compounds with deoxyribosides. Figure 1 shows the growth responses of strain 1417 to graded concentrations of several of these compounds.

MDDS contains a high proportion of yeast and of the extracellular products of yeast metabolism. It undergoes a mild alkaline hydrolysis with lime at about pH 10.5 during the process of manufacture and would doubtless contain deoxynucleosides, whose presence might account for most of its growth-promoting activity for strain 1417.

The organism was grown through many serial passages in chemically defined medium supplemented with thymidine, and appeared to have no residual indispensable requirement for unidentified nutrients. The cultures grew faster, however, and to about 20 % greater population, in basal medium supplemented with 10 % (v/v) of the MDDS extract than in the same basal medium supplemented with any one or all four of the deoxynucleosides. It is evident that the extract of MDDS contributes stimulatory nutrients other than the necessary deoxyribosides. It provides additional buffering which alone might have permitted greater total bacterial growth.

Requirements for exogenous deoxyribosides have been noted for several of the lactic acid bacteria, among them 'Thermobacterium acidophilus R 26', which was recommended by Hoff-Jørgensen (1951) for the microbiological assay of deoxyribonucleic acid and deoxyribonucleosides, and is presumably of the same species as that studied by us. In both organisms the requirement for deoxyribosides persists in the presence of vitamin B₁₂. *Lactobacillus delbrueckii* also shows an absolute requirement for deoxynucleosides; but in certain other species the requirement is non-specific and is not apparent in the presence of vitamin B₁₂ or enzymic digests of casein, or under reducing conditions induced by cysteine or ascorbic acid, or by growth in an atmosphere of hydrogen (Kitay, McNutt & Snell, 1949; Kitay, McNutt & Snell, 1950).

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A phage, $\phi\chi$, which attacks motile bacteria

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SUMMARY

A salmonella phage which attacks only flagellated bacteria (Sertic & Boulgakov, 1936*b*) has been studied. Tests with naturally occurring strains, and with artificial serotypes to which foreign H antigens had been transduced, have shown that sensitivity depends on the H antigen: bacteria with antigens of the *g*-complex are resistant, and with antigens *l...*, *e,h*, or Arizona 13, are sensitive only to appropriate host-range mutants. Tests with non-motile and motile variants of the same strains showed that paralysed (non-motile H) as well as non-flagellated bacteria are resistant and thus that the flagella must be active as well as of correct antigenic type. Where resistance was due to absence of suitable flagella, it was associated with impaired adsorption of phage. Removal of the flagella from a sensitive strain led to diminished adsorption; a similar result was obtained when the bacteria were artificially paralysed in various different ways. No adsorption to detached flagella was detected, probably because they were inactive. Adsorption of the phage led to immobilization and agglutination of the bacteria, probably by a direct effect on the flagella. Electron micrographs showed phage particles attached to flagella, and infection could evidently follow adsorption to distal parts of a flagellum. The genome of the infecting particle may perhaps reach the bacterial body by being injected into an active flagellum at the point of initial attachment, and then travelling inside the flagellum.

INTRODUCTION

The susceptibility of a bacterium to phage infection primarily depends on whether or not the phage can adsorb to specific bacterial receptors (see Nicolle, Jude & Diverneau, 1953). Sensitivity is usually determined by structures on the surface of the bacterial body, which have sometimes been identified as somatic antigens (e.g. Burnet, 1930), or more superficial envelope antigens such as the Vi antigen (Craigie & Brandon, 1936; Sertic & Boulgakov, 1936*a*; Nicolle, Rita & Huet, 1951). However, a phage whose host-range appeared to depend on the presence of flagella was described by Sertic & Boulgakov (1936*b*). This phage, first named VIII-113, and later χ (Sertic & Boulgakov, 1936*c*), lysed a motile strain of *Salmonella typhi*, H 901, but not its non-flagellated variant, O 901 (Felix, 1930). This phage also selected resistant bacteria from H 901 which were non-flagellated. Furthermore, it did not lyse H 901 growing on agar containing 0.2 % (w/v) phenol, known to prevent the development of flagella (Braun, 1918), and also did not produce plaques on

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strains carrying abundant Vi antigen, known to interfere with motility. Flagella are structurally distinct from components of the cell proper, and the flagellar (H) antigen does not extend over the surface of the bacterial body (Craigie, 1931; Stocker & Campbell, 1959); thus, if the flagella are concerned in adsorption, it seems likely that the flagella themselves must be the structures to which the phage adsorbs.

The phage under discussion was further studied by Schiff & Bornstein (1940) who claimed that it specifically attacked salmonella species with H antigen *d*, characteristic of *Salmonella typhi*. Rakieten & Bornstein (1941) reported the isolation of five other phages whose host-ranges were associated with flagella. Apart from reports of the use of the χ phage for selecting non-flagellated mutants from motile strains (Stocker, Zinder & Lederberg, 1953), the only other description was given by London (1958), who tentatively concluded that its site of attachment was associated with the basal granules of the flagella. London's object was to identify the chemical groupings involved in adsorption of the phage by treating the bacteria in various ways and by altering the ionic composition and pH of the medium. In so far as our experiments have overlapped, our results have been broadly the same, with one notable exception: the present experiments have shown that paralysed (i.e. flagellated but non-motile strains; Edwards, Moran & Bruner, 1946; Hirsch, 1947; Friewer & Leifson, 1952), as well as non-flagellated strains, are resistant to the χ phage, and that it adsorbs very poorly to paralysed bacteria. London obtained good adsorption to bacteria washed and suspended in dilute salt solutions, a treatment which, in the present experiments, caused sufficient loss of motility for adsorption to be greatly decreased.

METHODS

Buffers. M/15 phosphate buffer (pH 7.0) containing 0.1 M-NaCl, 10^{-3} M-MgSO₄, and 10^{-4} M-CaCl₂ with gelatin 0.001 % (w/v). McIlvaine's citrate phosphate buffers pH 2.2, 2.6 and 3.0.

Media. Broth was that routinely made in this department from Tryptone (Oxoid), 10 g.; Marmite 5 g.; sodium glycerophosphate, 10 g.; potassium lactate 50 % (w/v) solution, 5 ml.; MnSO₄·4H₂O, 0.02 g.; MgSO₄·7H₂O, 0.2 g.; FeSO₄·7H₂O₃ 0.02 g.; with water to 1 litre. The pH was adjusted to 7.2 and sterile glucose solution was added to 0.2 % (w/v).

Nutrient agar consisted of broth solidified with 1.25 % (w/v) Davis N. Z. agar. In phage titrations by the overlay method, the bases and overlays contained 0.6 and 0.3 % (w/v) agar, respectively. Semi-solid motility medium (Edwards & Ewing, 1955) was dispensed in 8 ml. volumes in 2 in. diameter Petri dishes. All cultures were incubated at 37° without aeration.

Phage. The χ phage was originally obtained from Dr N. Boulgakov by Dr B. A. D. Stocker in 1952, and was usually grown and assayed on *Salmonella abortus-equi*, National Collection of Type Cultures (NCTC) 5727, a species not pathogenic for man. Before this strain had been found to be as good an indicator, Sertic & Boulgakov's usual propagating strain, *S. typhi* var Rough (now called SW 540) was used. Stocks were made by the overlay method (Swanstrom & Adams, 1951) with overlays and bases containing 0.3 and 1.25 % (w/v) agar, respectively. The homogenized overlays were clarified by centrifugation and the remaining bacteria were killed by heating at 56°–60° for 30 min. Titres of 5×10^{10} – 2×10^{11} plaque-forming particles (p.f.p.)/ml.

were readily obtained. In tests with a limited number of strains, the efficiencies of plating of stocks made on the two hosts, NCTC 5727 and SW 540, were similar.

Stocks of host-range variants of the χ phage. When the χ phage plated with low efficiency on a strain, one of the few plaques produced was diluted and replated with the strain to purify the phage, and a single plaque was then picked and grown with the strain concerned to make the variant stock. Stocks grown on NCTC 5727 were made from single plaques produced by the variant stocks on this strain. *Salmonella abortus-equi* strain NCTC 5727 was tested for possible lysogenicity with a phage whose presence in stocks might have been mistaken for a low efficiency of plating of the χ phage itself: supernatant fluids from cultures of NCTC 5727 treated in the same way as the χ phage stocks produced no plaques on any of the strains concerned. Nor did they produce plaques on strains of *Salmonella gallinarum*, a species sensitive to a wide range of salmonella phages (Dr E. S. Anderson, personal communication). To be sure that the phages with altered host-range were in fact variants of the χ phage, they were tested for neutralization by antiserum prepared against the wild-type phage: all were neutralized at the same rate as this was. Their activity against the strains which had selected them was neutralized to the same extent as their activity against NCTC 5727. They were also tested with motile and non-motile (O or paralysed) forms of a few different salmonella strains, and, like the wild-type phage, they attacked a strain only when it was motile.

Phage titrations. (1) Agar layer method. Satisfactory titrations giving consistent results were obtained by this method with *Salmonella abortus-equi*, NCTC 5727 or *S. typhi*, SW 540 as indicator strain, provided the volume of the overlay was not more than 2.5 ml. and the plates were kept strictly level. *Salmonella* strain NCTC 5727 tended to become less motile on subculture and the plaques then became hazy and small, but with passage of the indicator strain through semi-solid medium, the plaques again became larger and clearer.

(2) Surface method. Satisfactory plaques were usually obtained when drops of phage stocks were spotted on plates spread with indicator strain. Pipettes delivering drops of 0.02 ml. were used, and in general serial 10-fold dilutions were plated. With *Salmonella abortus-equi* strain NCTC 5727 as indicator, the same numbers of plaques were obtained by either the overlay or the surface method.

The agar layer method was suitable for use with strains of *Salmonella abortus-equi* or *S. typhi*; with other species, the number of plaques was greatly decreased when the concentration of agar in the overlay was high enough to give discrete plaques.

Where the titre of the phage is given, it refers to the number of plaque-forming particles (p.f.p.) on *Salmonella abortus-equi* strain NCTC 5727. The efficiency of plating (e.o.p.) on other strains was calculated using the titre on strain NCTC 5727 as denominator.

Total phage counts by fluorescence microscopy. Suspensions of phage were mixed with a bacterial suspension of known concentration. The mixture was examined by fluorescence microscopy (Anderson, Armstrong & Niven, 1959; Anderson, 1957) and the phage count estimated from the ratio of phage particles to bacteria.

Bacteria. The antigenic formulae for *Salmonella* are written according to the Kauffmann-White Scheme and no account has been taken of minor antigenic relationships or differences omitted from this scheme. For example, 1, 4, 5, 12:b:1,2, the formula for *Salmonella paratyphi B*, indicates that the strain has somatic (O)

antigens 1, 4, 5, 12 and flagellar (H) antigens *b* and 1,2, either of which may be present since they are subject to the rapid mutation and back-mutation known as phase variation (Andrewes, 1922, 1925). The formula for *S. typhi* is 9, 12 (Vi):*d*:-, and the formula for *S. abortus-equi* is 4, 12:-*e,n,x*; these species are monophasic in phase 1 and phase 2, respectively.

The strains examined came from several sources which are indicated by the letters preceding their numbers: e.g. NCTC (National Collection of Type Cultures, Colindale Avenue, London, N.W. 9); SW, SL, SR, SY, or LT (Guinness-Lister Unit, Lister Institute of Preventive Medicine, Chelsea Bridge Road, London, S.W. 1); some of these strains were described by Stocker *et al.* (1953) or by Lederberg & Edwards (1953); A (Dr E. S. Anderson, Central Enteric Reference Laboratory, Colindale Avenue, London, N.W. 9). Other strains were provided by Dr Joan Taylor (Salmonella Reference Laboratory, Colindale), and by Dr P. R. Edwards (Communicable Disease Center, Chamblee, Georgia, U.S.A.). One of the latter is mentioned individually preceded by the letter E.

Tests were made on as many non-motile strains as possible, some of which were non-flagellated while others were 'paralysed', i.e. they possessed inactive flagella of normal serological and morphological structure. Examination of stained preparations and electron microscopy of the paralysed strains used here revealed no differences from normal motile strains either in number or appearance of flagella.

Motile variants of some non-motile strains were sometimes provided; otherwise these were isolated when possible by selection of mutants in semi-solid medium or by transduction using phage P22 (Stocker *et al.* 1953) grown on a motile strain, either *Salmonella typhimurium* strain LT2 (1, 4, 5, 12:*i*:1,2), or *S. enteritidis* strain SL 431(1, 9, 12:*g,m*:-).

One strain, SJ 30, isolated as a spontaneous mutant from *Salmonella abortus-equi* NCTC 5727 by Dr Tetsuo Iino, had 'curly' flagella (Leifson, 1951; Leifson & Hugh, 1953) which have the usual sinuous form in fixed preparations, but whose wavelength is less than normal (1.5 μ as compared with 2.6 μ in the case of SJ 30 and NCTC 5727; Dr Iino, personal communication). Strain SJ 30 showed no translational motility, but very vigorous jerking and rotation; it had also a very marked tendency to clump, as has been observed with other bacteria having flagella of this type. Motile back-mutants with 'normal' flagella lose this tendency to clump.

Synthetic strains with foreign H antigens introduced by transduction (Lederberg & Edwards, 1953; Lederberg & Iino, 1956). Most of these strains came from the Guinness-Lister Unit. The phase 1 antigens *l*, *z*₁₃, *z*₁₀ and *z*, were introduced into *Salmonella typhi*, H 901, from *S. napoli* (1, 9, 12:*l,z*₁₃:*e,n,x*) NCTC 6853, *S. ituri* (1, 4, 12:*z*₁₀:1,5) NCTC 8275 and *S. shubra* (4, 5, 12:*z*:1,2) SL 652, respectively. The phase 2 antigen 1,7 was introduced into *S. abortus-equi* NCTC 5727 from *S. kaapstad* (4, 12:*e,h*:1,7) SL136. Phage P22 was used as transducing phage, and bacteria of the recipient strain with the new antigen were isolated by means of Edwards motility medium containing either anti-*d* or anti-*e,n,x* serum. In the course of unsuccessful attempts to introduce other phase 2 antigens into NCTC 5727, bacteria of this strain were isolated in phase 1, with antigen *a*, which is usually suppressed (Edwards & Bruner, 1939). Attempts to introduce other antigens (*z*₂₉; *z*₃₈; *k*; *z*₆; 1,6; *y*) into these hosts failed, usually because no strains naturally carrying these antigens were found which were sensitive to phage P22.

Selection of Vi-negative variants of Salmonella typhi. A pool of Vi phages I-VII (kindly provided by Dr E. S. Anderson) was spotted on lawns of the Vi-positive strains of *Salmonella typhi*. Resistant bacteria selected by this phage pool have lost the Vi antigen (Dr E. S. Anderson, personal communication). Single colonies obtained by subcultivation from the area of lysis were isolated, and identified as Vi-negative when they were resistant to the phage pool.

Selection of monophasic populations from diphasic strains. Whenever possible, suitable colonies were identified by slide agglutination; when this failed the strains were passed through semi-solid medium containing H antiserum to the unwanted phase.

Bacterial counts. Viable counts were made by spreading 0.2 ml. samples from dilutions of culture on the surface of nutrient agar plates. Total counts were done in a Helber counting chamber.

The proportion of motile bacteria. This was estimated by dark-field microscopy of broth cultures, usually with an ordinary slide and coverslip. Repeated counts of the same suspension gave comparable results. When special accuracy was needed, a Helber chamber was used, the estimate being based on separate counts of the total number of bacteria and of the number of motile or non-motile organisms (whichever was in a minority). Motility was recorded either as 'translational' when the organisms progressed across the field, or as 'rotational' when they rotated or tumbled about one spot.

Sensitivity tests. A loopful of an overnight broth culture of the test strain was spread over 1/3 of a nutrient agar plate. Drops of 0.02 ml. of three dilutions of the χ phage were spotted on this lawn, the drops containing $c. 5 \times 10^8$, $c. 5 \times 10^3$ and $c. 5 \times 10^1$ p.f.p., respectively. After overnight incubation, the degree of clearing by the most concentrated suspension was recorded as + + +, + + or +, or (+) where the clearing was so slight as to be seen only when the plate was viewed obliquely. The effects produced by the other dilutions were recorded as +, semi-confluent plaques, or as an approximate estimate of the number of plaques with a brief description of their appearance. *Salmonella abortus-equi* strain NCTC 5727 was always included as a control. The conditions for plaque formation appeared to be more critical with some strains than with others, and the tests had then to be repeated several times to obtain an unambiguous result. In a few tests with such strains with 0.6 % (w/v) agar, the strains did not appear to be more sensitive than on the higher concentration.

Almost all the motile strains were passed through motility medium before testing, and with all strains, the overnight cultures which provided the inocula for sensitivity tests were examined for motility: all the strains classified as motile showed more than 50 % of motile bacteria; almost all showed more than 80 %. The non-motile strains were tested for phage-sensitivity in parallel with their motile relatives. The naturally occurring motile strains were not routinely checked for antigenic structure in these tests; but the synthetic strains with foreign antigens and the motile derivatives of non-motile strains were always tested by slide agglutination with H antisera. The non-motile strains were also tested in this way to determine whether they were non-flagellated or paralysed.

Isolation of stable χ phage-resistant variants of sensitive strains. Twenty-six salmonella strains were chosen, and an attempt was made to isolate 6 independent

resistant variants from each by exposing 6 separate single colony isolates to the phage on agar. Resistant colonies from the patch of lysis were purified by 4 successive single colony isolations. Many isolates reverted to sensitivity during purification and were discarded. When none of 20 colonies from a lysed patch remained resistant, the original strain was grown in broth with the phage, but this was little more successful than culture on agar. Six resistant variants came from the collection of the Guinness-Lister Unit.

Determination of phage adsorption. (1) Measurement of unadsorbed phage. Cultures in the late exponential phase of growth were used when the viable count was about $2-5 \times 10^8$ organisms/ml. Except where the effect of multiplicity was specifically measured, the ratio of phage (p.f.p.) to bacteria was always less than 0.1. After 5, 10 or 20 min. at 37° , the mixtures or their dilutions were centrifuged and the supernatant fluids titrated for free phage. (2) Measurement of infected bacteria. In these experiments, the ratio of phage to bacteria was 0.05 or less. The mixtures were diluted into antiphage serum at a concentration which inactivated more than 99% of free phage in 2 min. After 5 min., they were further diluted and plated with *Salmonella abortus-equi* strain NCTC 5727.

Electron microscopy. (1) Fixed, platinum-iridium-shadowed preparations. The material consisted of phage bacterium mixtures containing 10-40 p.f.p./bacterium; after 0.5-15 min., formalin was added to 10% (v/v). After standing overnight at room temperature, the mixtures were thoroughly washed in distilled water. It was calculated that, with the highest concentration of phage used, the washing was sufficient to leave less than 1 unadsorbed phage particle for every 15 bacteria. The specimens for electron microscopy were prepared and the electron microscopy performed by Mrs H. Ozeki at the Chester Beatty Research Institute. Phage stocks alone and a few bacterial strains without phage were also examined in this way. (2) Preparations negatively-stained with phosphotungstic acid (Brenner *et al.* 1959) were kindly made and examined by Dr E. H. Mercer and Dr M. Birbeck at the Chester Beatty Research Institute, using unfixed material consisting of phage alone or freshly made mixtures of phage and bacteria.

Blending. An M.S.E. Blendor (Measuring and Scientific Equipment Ltd., Spenser Street, London, S.W. 1) was used to stir 10-15 ml. volumes in 25 ml. 'Universal' screw-capped containers. With more than 1.5-2 min. blending of a bacterial suspension, no further decrease in the proportion of motile bacteria was observed. Blending for 2 min. did not alter the titre of a phage stock..

Preparation of detached flagella. Large crops of bacteria were grown on trays of nutrient agar incubated overnight at 37° . The growth was washed off in distilled water with minimal rubbing of the bacteria against the agar surface. The suspension was next treated in the M.S.E. Blendor in 10 ml. volumes followed by the removal of most of the bacterial bodies by centrifugation at 1100 g. The supernatant fluid was then centrifuged in a Spinco model L ultra-centrifuge at 20,000 g for 1 hr. to deposit the flagella. This deposit was resuspended in 30 ml. distilled water and centrifuged at 1100 g and the resulting supernatant fluid again centrifuged at 20,000 g. The deposit of flagella finally obtained was resuspended in about 2 ml. distilled water to give a grey opalescent suspension. When any bacterial bodies were seen microscopically in this suspension, they were removed by further low speed centrifugation.

Antiphage sera. Rabbits were injected intravenously with phage stocks grown on *Salmonella abortus-equi* strain NCTC 5727 and having titres of $c. 2 \times 10^{10}$ p.f.p./ml. The antisera were absorbed with the propagating strain before use.

Antibacterial sera. In most cases, slide agglutination alone was done to determine the flagellar antigens of a strain. Most of these tests were made with antisera prepared by the Standards Laboratory, Central Public Health Laboratory, Colindale. Antisera for inclusion in semi-solid motility medium were prepared here by inoculating rabbits intravenously with overnight broth cultures sterilized by heating for 1 hr. at 56° ; where possible, monophasic strains were used.

RESULTS

General characteristics of the χ phage

The plaques of the χ phage on *Salmonella abortus-equi* NCTC 5727 and on *S. typhi* SW 540 with optimal plating conditions were $c. 1$ mm. in diameter, punched-out in appearance and containing only a little bacterial growth, usually in the form of a granular film. The conditions for plating were rather critical; small changes might cause the plaques to become either large, smeary and filled with growth, or so small as to be scarcely visible and greatly decreased in number. No plaque-type variants were obtained; differences in plaque appearance which were observed were accidental and were not inherited. Plaques similar to those produced in an agar layer, but usually rather smaller, were produced when the phage was spotted on full-strength (1.25% w/v) agar plates spread with strains NCTC 5727 or SW 540. There was no indication that the χ phage could lysogenize.

Almost clear plaques were also seen on other strains of *Salmonella abortus-equi* and on most sensitive strains of *S. typhi*, and the patch of lysis produced by drops of concentrated phage was similarly covered with only a thin film of bacterial growth. On the sensitive strains of other species the plaques and patches were usually much less thoroughly cleared and were often extremely shallow. Broth cultures were never cleared, in agreement with the findings of Dr Boulgakov (personal communication to Dr B. A. D. Stocker) and of Rakieten & Bornstein (1941) with their flagellar phages.

In one-step growth experiments, using *Salmonella abortus-equi* NCTC 5727 as host with a low multiplicity of infection, the minimum latent period was 55–60 min. and after the curve flattened $c. 90$ min. after infection, the average burst size was $c. 200$. With a multiplicity greater than one, the minimum latent period was about the same, but the burst size could not be estimated because of bacterial clumping, which will be described later.

Lysates with titres of $2-5 \times 10^{11}$ p.f.p./ml. were readily obtained. Filtrates through membrane filters were almost without activity; only $2.5 \times 10^{-8} - 5 \times 10^{-7}$ of the original number of p.f.p. were obtained. Filtrates through Seitz filters were similarly inactive; less than 2×10^{-7} p.f.p. were present. With sintered glass filters, the filtrates contained $c. 5\%$ of the original number of p.f.p. The phage stocks which were used were freed from living bacteria by heating at $56^\circ-60^\circ$ for 30 min., although with 30 min. at 60° , the p.f.p. were decreased to 35–50%, and with 1 hr. to 12–20% of the original values.

The χ phage is about fifteen times as resistant to ultraviolet radiation as is phage T2 (Adams, 1959).

The χ phage was a good antigen; neutralizing sera with K values of 3500 were easily obtained.

Particles of the χ phage are tadpole-shaped. Electron micrographs of preparations negatively-stained with phosphotungstic acid showed a head about 675 Å in diameter and a long tail about 2300 Å in length and 125 Å in width (Pl. 1, figs. 1, 2). The tail showed fine transverse striations like those of phage T2 (Brenner *et al.* 1959), and in some particles the head appeared hexagonal in shape. A few particles with collapsed heads were seen in untreated phage stocks, but many more of these were seen in preparations which had been heated at 56°–60° for 30 min. When the phage had been deposited by high-speed centrifugation (16,000 g for 90 min.), 99.9 % of the original p.f.p. were inactivated and the heads of almost all the particles appeared to be collapsed.

The χ phage evidently multiplies intracellularly like other phages, for electron micrographs of sections of infected bacteria in plaques, kindly made by Dr E. H. Mercer using the technique he developed for phage T2 (Mercer, 1959), showed dense phage-like structures like those seen with phage T2 and thought to be the DNA of immature phage heads (Kellenberger, Séchaud & Ryter, 1959; Mercer, 1959). Also, the sequence of events after infection as observed by fluorescence microscopy was not qualitatively different from that seen with other phages (Anderson, Armstrong & Niven, 1959; Dr E. S. Anderson, personal communication). Thus, there is no reason to think that the χ phage is an agent active only against the flagella, as suggested by Rakieten & Bornstein (1941), a theory which would, moreover, presuppose that this phage constituted a completely new kind of bacterial parasite.

The susceptibility of highly motile strains of naturally occurring serotypes

The χ phage was tested against 524 naturally occurring salmonella strains of various serotypes to investigate the possible influence of antigenic structure on its host-range. The strains tested were highly motile on the assumption, taken from previous authors, that sensitivity was correlated with the presence of flagella. The identities of the strains are listed in the footnote to Table 1, which shows the results of the tests. The appearance of the plaques, i.e. size and degree of clearing, varied considerably from strain to strain, but with most strains on which any plaques could be seen, the efficiency of plating (e.o.p.) was over 0.05. Strains on which the phage had a much lower e.o.p. were:

(a) Nine strains of *Salmonella typhi*, (9, 12; d:–) comprising all of 8 strains of Vi-phage type 32 tested and the only strain of type B2; the former were completely resistant while Vi-positive. Plaques on Vi-negative derivatives of these strains were not regularly observed; when present, they were minute and the e.o.p. was $c. 10^{-4}$.

(b) all of 4 sensitive strains of *S. paratyphi A* (1, 2, 12; a:–), A. A203, A. BA528, NCTC 13, and NCTC 9452, the e.o.p. being 10^{-4} .

(c) one strain of *S. paratyphi B* (1, 4, 5, 12; b:–) SW 543 S (e.o.p. 10^{-4}), and 2 strains of *S. typhimurium* (1, 4, 5, 12; i:1,2), SW 964 S and SL 653 (e.o.p. 10^{-6}).

Table 1. *Host-range of the χ phage with highly motile naturally occurring Salmonella strains of various serotypes*

Five hundred and twenty four Salmonella strains were tested, which comprised 104 strains of *S. typhi* with Vi antigen, 13 strains of this species without Vi antigen, (37 of the Vi-positive strains were also tested in the Vi-negative state); 61 strains of other species in O Group D, 46 strains of *S. paratyphi* B, 2 of which were fixed in phase 1 with H antigen *b*, and 1 of which was also fixed in phase 1 although it had H antigen *I*, 2; 61 strains of *S. typhimurium*, 1 fixed in phase 1 and 3 fixed in phase 2; 74 strains of other species in O Group B; 13 strains of *S. paratyphi* A; 3 strains of *S. paratyphi* C; 33 strains of other species in O Group C 1; 21 strains in O Group C 2; 13 strains in O Group E 1; 9 strains in O Group E 2; 5 strains in O Group E 3; 6 strains in O Group E 4; 5 strains in O Group F; 8 strains in O Group G; 8 strains in O Group H; 6 strains in O Group I; and 35 strains in the further O Groups, one or a few from each Group.

The method of testing is described in the text. A strain was recorded as:

(1) *Fully sensitive* if (a) the undiluted phage stock produced clearing which was marked, or at least easily seen, and (b) the 10⁻⁵ dilution produced semi-confluent lysis, or at least 50-100 plaques, showing that the e.o.p. was not lower than 0.01.

(2) *Slightly sensitive* if the undiluted stock only produced slight thinning (recorded as (+)), and its dilutions had no effect.

(3) *Resistant* if the undiluted stock and its dilutions had no effect.

A few strains fell into two further categories: (4) those on which the undiluted stock produced marked clearing but the 10⁻⁵ dilution had no effect; more precise titrations showed that the phage had a low e.o.p. on these strains; and (5) strains on which the

undiluted stock produced only discrete plaques. In the table, the denominator represents the number of strains of the particular serotype tested; in the numerator, the number outside brackets is the number of fully sensitive strains, the number in brackets is the number of slightly sensitive strains, the number preceded by C is the number cleared by the undiluted phage but showing no effect with the 10⁻⁵ dilution, and the number preceded by P is the number on which the undiluted phage, gave only discrete plaques. The difference between numerator and denominator gives the number of resistant strains. The diphasic strains, Sections 2 and 3, are represented twice, once under the H antigen of each phase.

Somatic group	Section 1. Monophasic strains with flagellar antigens																Section 2. Diphasic strains with flagellar antigens (also occur in monophasic strains)																Section 3. Diphasic strains with flagellar antigens (occur only in diphasic strains)																Total strain	
	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>	<i>i</i>	<i>z</i> ₄ <i>z</i> ₂₃	<i>z</i> ₄ <i>z</i> ₂₄	<i>z</i> ₂₆	<i>z</i> ₂₉	<i>z</i> ₃₈	<i>g</i> ...	<i>m, t</i>	<i>e, n, x</i>	<i>1, 2</i>	<i>1, 5</i>	<i>l, v</i>	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>	<i>i</i>	<i>z</i> ₄ <i>z</i> ₂₃	<i>z</i> ₂₉	<i>g</i> ...	<i>m, t</i>	<i>e, n, x</i>	<i>1, 2</i>	<i>1, 5</i>	<i>l, v</i>	<i>l, w</i>	<i>l, z</i> ₁₃	<i>k</i>	<i>r</i>	<i>y</i>	<i>e, h</i>	<i>z</i>	<i>z</i> ₁₉	<i>e, n, z</i> ₁₅	<i>1, 6</i>	<i>1, 7</i>	<i>z</i> ₆									
A	C 4(1) 13	C 4(1)/13	A				
B	.	C 1 2	.	.	1/1	.	.	.	(1)/4	.	0/10	0/1	4/4	3/4	.	0/1	.	.	28(12) 47	0/2	3/4	45 C 2 (8)/60	(4)/10	76 C 2 (25)/111	(2)/5	1(5)/10	2(1)/10	0/1	1(1)/2	1(1)/4	(2)/3	(1)/9	2(1)/6	3(1)/4	2(3)/7	1(1)/2	(1)/6	3/5	92 C 3(34)/181	B						
C 1	0/4	0/1	0/2	0/4	.	.	2(1)/5	.	1(1)/2	1(1)/3	4/4	(1)/3	.	(1)/1	(1)/1	4 P 1(1) 8	0/1	(1)/4	P 1/1	.	.	.	(2)/4	.	.	1(1)/5	(1)/1	(1)/1	1/1	8 P 1(7)/36	C 1							
C 2	.	.	.	(1)/1 33 C 9	.	(1)/1	1/2	.	.	.	0/1	.	.	2/2	.	.	1/1	(2)/2	1/1	1(1)/3	(2)/3	1/1	(1)/5	(1)/1	(2)/2	0/1	.	.	(1)/1	1/1	0/3	.	1/1	(1)/1	(1)/1	(3)/4	5(9)/21	C 2								
D	.	.	.	(5)/50 82 C 1	.	0/1	.	.	(3)/3	(2)/4	0/19	1/1	1(1)/2	1(1)/3	1(1)/3	.	.	0/2	0/1	.	0/6	1/2	2/9	1(1)/8	0/1	1/4	0/3	1/1	.	.	0/3	2/3	.	1(1)/4	2(1)/3	1/3	3(2)/7	42 C 9(14)/111	D						
D Vi ⁺	.	.	.	(7)/104	82 C 1(7)/104	D Vi ⁺						
E 1	1/1	.	.	0/1	1/1	0/1	1(1)/5	0/1	0/1	0/1	(1)/1	.	(1)/2	0/3	0/1	0/1	.	0/1	0/2	(1)/1	2(2)/13	E 1						
E 2	0/1	(1)/2	0/1	0/2	(1)/1	.	.	0/1	0/4	0/1	.	0/1	0/1	(1)/4	(1)/1	2(2)/9	E 2						
E 3	0/3	(1)/1	0/1	P 1/3	P 1/1	.	.	.	P 1/3	.	P 1/1	.	P 2/5	E 3						
E 4	0/1	1/1	.	.	(1)/1	0/1	0/1	(1)/1	(1)/2	2(2)/6	E 4						
F	0/1	0/1	0/1	0/1	(1)/1	1(2)/3	(1)/1	(1)/1	1(3)/5	F						
G	.	(1) 1	0/1	.	.	0/1	1/1	0/1	.	(1)/1	.	.	0/1	.	0/1	.	0/1	0/1	(1)/1	(1)/1	2(2)/8	G						
H	1/1	0/1	.	.	.	0/1	0/1	1/1	0/1	.	(1)/1	1(1)/2	.	0/1	.	0/2	.	.	0/2	0/1	1/1	2(1)/8	H						
I	0/1	2 P 1(1)/6	I						
17	1/2	1/1	0/1	3/4	17					
18	0/1	2(1)/5	18					
21	0/1	3/4	21					
28	0/1	1(2)/5	28				
30	0/1	(1)/1	.	.	(1)/1	(1)/1	1(1)/5	30					
35	.	.	0/1	.	.	1/1	0/2	(1)/1	(1)/1	1(1)/3	35					
38	0/1	38				
39	0/1	39				
40	1(1)/2	1(1)/2	40					
41	2/2	2/2	41				
42	0/1	(1)/1	(1)/1	(1)/1	(1)/2	42					
43	(1)/2	43				
44	0/1	44				
45	0/2	45				
Total strains	C 4(1) /13	C 1(1) /3	0/1	100 C 9 (6)/118*	1/1	5(1)/8	2/5	1/2	(4)/12	(2)/5	0/44	(1)/7	4/4	5/6	2(1)/5	0/1	4(2)/7	31(20) /61	6(11)/11	5(5)/15	45 C 2 (12)/66	(1)/1	0/3	0/2	0/1	2(9)/28	81 C 2 (28)/121	13 P 3 (11)/49	3 P 1 (10)/28	3(2)/25	P 1(1)/7	4(2)/8	2(5)/9	1 P 1(5) /12	P 1(3) /28	5(2)/4	3 P 1(1) /8	5(9)/22	3 P 2(4) /12	1(5)/20	7(7)/20									

* 37 strains of *S. typhi* were tested in both the Vi⁺ and Vi⁻ state, see pp.261-262.

Strains on which the undiluted phage stock produced only discrete plaques were: *S. ness-ziona* (6, 7:l,z₁₃:1,5), NCTC 8717; *S. shanghai* (16:l,v:1,6), SL 651; *S. kasenyi* (38:e,h:1,5), NCTC 8278, (the plaques were on a background of slight clearing of degree (+), and dilutions of the phage sometimes also produced a much larger number of very indistinct plaques on this strain); *S. thomasville* (3, 15, 34:y:1,5), NCTC 9896; *S. harrisonburg* (3, 15, 34:z₁₀:1,6), NCTC 8258. A few of the strains which were classed as slightly sensitive may really have fallen into this category, as there was sometimes a suggestion of plaques in the partially cleared patch. These did not always appear on repeated testing and attempts to isolate phage from them which would give more definite plaques on the strain failed.

These host-range tests showed the following results:

(1) The H antigens could determine the sensitivity of a strain, for all strains which carried antigen *g* were resistant, regardless of whether this antigen was present as *g,m*, *g,p*, *g,s,t*, etc., (one slightly sensitive strain with antigen *m,t* constituted a weak exception to the resistance of strains with antigens of the 'g complex'). Most strains with *g*-related antigens are monophasic, but there are a few diphasic serotypes (Kauffmann & Henning, 1952; Douglas, Taylor & McMath, 1951). Three such diphasic serotypes were tested and were found to be predominantly in the *g* phase and resistant when first tested; but by passing them through motility medium with antiserum it was possible to isolate bacteria in the alternative phase. When these strains were tested with the χ phage, one (with antigen *e,n,x*) was fully sensitive, and the other two (with antigens *e,n,x* and *1,5*, respectively) gave a (+) reaction.

The χ phage was also tested on 20 strains of the Arizona group (Edwards & Ewing, 1955; Edwards, Fife & Ramsey, 1959) carrying various O and H antigens. Eleven strains were sensitive. Some Arizona H antigens are shared with *Salmonella*; in particular, H antigen 13 cross-reacts with salmonella H antigen *g* (Edwards & Ewing, 1955; Edwards *et al.* 1959). Four of five Arizona strains carrying antigen 13 which were tested were χ phage-resistant, and the fifth, NCTC 7318, (O antigen 9; H antigen 13, 15) showed a few discrete plaques with the undiluted phage.

Strains carrying most of the other salmonella H antigens might be either sensitive or resistant, showing that susceptibility is not governed solely by H antigenic type.

(2) The somatic antigen probably did not determine sensitivity, for strains belonging to many different O Groups, as well as rough strains which had lost their O antigen (e.g. *Salmonella typhi* SW 540), could be sensitive. None of the strains belonging to O Groups E3, E4 or G which were tested were fully sensitive. Undiluted phage produced only discrete plaques on *S. harrisonburg* NCTC 8258 and *S. thomasville* NCTC 9896, belonging to Group E3. The results with some other somatic antigens do not carry much weight since so few strains were tested.

(3) The most sensitive species were *Salmonella typhi* and *S. abortus-equi*, both in the completeness of clearing in individual plaques and in patches produced by concentrated phage, and also in the proportion of strains found to be sensitive. One hundred and seventeen strains of *S. typhi* were tested, including 104 Vi-positive strains from 67 different Vi-phage types (Craigie & Yen, 1938), and 13 Vi-negative strains. Of 104 Vi-positive strains 82 fell into the fully sensitive category, but only 67 showed maximum clearing (+++), leaving 37 strains showing various degrees of resistance. Of these 37 strains, 21 owed their resistance either to the presence of the Vi antigen, as found by previous authors, or to poor motility. When the sensitivity

of the 117 strains was assessed, taking into account the reaction of these 37 strains either after loss of the Vi antigen or after passage through motility medium, only 17 of the 117 tested were not fully sensitive, with + + + clearing by undiluted phage.

Effect of Vi-phage type. The Vi-phage type of a strain was correlated with its reaction to the χ phage since: (a) 3/3 strains of Vi-phage type D4 were totally resistant; (b) the 5 strains giving a (+) reaction comprised 4/4 strains of type M (2 of type M1 and 1 each of types M2 and M3) and 1/1 strain of type 37; (c) the 9 strains showing plaques only irregularly and with low e.o.p. comprised 8/8 strains of type 32 and 1/1 strain of type B2.

Vi-phage typing depends on specific modifications in the host-range of Vi phage II (Anderson & Felix, 1953), but of the 67 types, only types D4 and M are resistant to all the *Salmonella typhi* Vi phages (I-VII) except phage III (Dr E. S. Anderson, personal communication). A correlation between χ phage resistance and Vi-phage type, especially if the type is rarely isolated and is restricted to a particular locality, might have no more significance than repeated tests on the same strain, but type D1 (4/4 strains), which is closely related to type D4, was fully sensitive to the χ phage. Clones of type D1 frequently arise in cultures of type D4, and such clones are sensitive to the χ phage while the parent culture remains resistant (Dr E. S. Anderson, personal communication). The change from type D4 to type D1 is not associated with loss of one of the recognized type-determining phages; types D4 and D1 carry the same type-determining phage, and type A (which is sensitive to all the adapted preparations of Vi phage II) remained fully sensitive to the χ phage after lysogenization with this phage isolated from either a type D1 strain or a type D4 strain. Thus the resistance of type D4 to the χ phage is not due to lysogenicity with a type-determining phage. One strain of type D4 absorbed the χ phage rapidly; therefore resistance in this strain at least was not due to failure of attachment.

The susceptibility of highly motile strains with artificial serotypes

The relation between χ phage sensitivity and H antigenic type was further examined by testing sets of artificial derivatives with differing H antigens prepared from single parental strains by transduction. The results in Table 2 allow the following conclusions.

(1) When a strain was originally sensitive, it kept its sensitivity with most of the foreign H antigens. The degree of clearing produced by the undiluted phage stock and the appearance of individual plaques were characteristic of the strain, and not of the H antigen it carried. Occasionally, a change of H antigen brought with it a small change in e.o.p. (c. five-fold) but without extensive tests it is impossible to be sure that this difference was greater than would have been found in tests on different clones of the same strain.

The low e.o.p. (c. 10^{-4}) of the phage on strains of *Salmonella paratyphi* A was characteristic of the bacterium and not of the H antigen *a*, for the phage still plated with an e.o.p. of 10^{-4} on derivatives of *S. paratyphi* A, strain A. 17689, in which antigens *i* or *r* had been substituted for *a*. SL 508 (*S. typhi* SY 79 with antigen *a* transduced from *S. abortus-equi* NCTC 5727) was fully sensitive, as was NCTC 5727 itself with antigen *a* in phase 1 which is normally suppressed (Edwards & Bruner, 1939).

Table 2. Sensitivity of synthetic *Salmonella* strains with various flagellar antigens

Strain	Antigen	Sensitivity	Donor of antigen	Sensitivity of donor*
<i>S. typhi</i>				
SY 79	<i>d</i> :-	+	.	.
Derivatives				
SW 520	<i>i</i> :-	+	<i>S. typhimurium</i> LT 7	+
SL 502	<i>b</i> :-	+	<i>S. abony</i> SW 803	-
SL 504	<i>r</i> :-	+	<i>S. heidelberg</i> SL 142	+
SL 505	<i>c</i> :-	+	<i>S. altendorf</i> SL 137	-
SL 508	<i>a</i> :-	+	<i>S. abortus-equi</i> NCTC 5727	+
SL 509	<i>l</i> , <i>z</i> :-	+	<i>S. paratyphi B</i> SW 546†	+
SL 506	<i>e</i> , <i>h</i> :-	e.o.p. $10^{-6}\frac{1}{2}$	<i>S. chester</i> SL 139	-
SL 507	<i>e</i> , <i>h</i> :-	e.o.p. $10^{-6}\frac{1}{2}$	<i>S. kaapstad</i> SL 136	-
SL 503	<i>g</i> , <i>p</i> :-	-	<i>S. dublin</i> SW 553 (O)	-
SL 510	<i>g</i> , <i>m</i> , <i>s</i> :-	-	<i>S. hato</i> NCTC 9899	-
SL 511	<i>g</i> , <i>m</i> :-	-	<i>S. enteritidis</i> NCTC 4196	-
<i>S. typhi</i>				
SW 537 (H901)	<i>d</i> :-	+	.	.
Derivatives				
SW 569	<i>i</i> :-	+	<i>S. typhimurium</i> LT 2	+
SW 902	<i>c</i> :-	+	<i>S. altendorf</i> SW 825	-
SW 537. <i>z</i>	<i>z</i> :-	+	<i>S. shubra</i> SL 652	(+)
SW 537. <i>z</i> ₁₀	<i>z</i> ₁₀ :-	+	<i>S. ituri</i> NCTC 8275	(+)
SW 668	<i>e</i> , <i>h</i> :-	e.o.p. $10^{-6}\frac{1}{2}$	<i>S. sandiego</i> SW 718	.
SW 537. <i>l</i> , <i>z</i> ₁₃	<i>l</i> , <i>z</i> ₁₃ :-	e.o.p. 10^{-7}	<i>S. napoli</i> NCTC 6853	.
SW 667	<i>g</i> , <i>p</i> :-	-	<i>S. dublin</i> SW 553 (O)	-
<i>S. abortus-equi</i>				
NCTC 5727	-: <i>e</i> , <i>n</i> , <i>x</i>	+	.	.
Derivative				
NCTC 5727. <i>l</i> , <i>7</i>	-: <i>l</i> , <i>7</i>	+	<i>S. kaapstad</i> SL 136	-
<i>S. paratyphi A</i>				
A 17689	O strain	(-)	.	.
Derivatives				
SL 31	<i>i</i> :-	e.o.p. 10^{-4}	<i>S. typhimurium</i> LT 2	+
SL 37	<i>r</i> :-	e.o.p. 10^{-4}	<i>S. heidelberg</i> SL 28 (O)	+
<i>S. paratyphi B</i> (monophasic)				
SW 543	O strain	(-)	.	.
Derivatives				
SW 543 swarm	<i>b</i> :-	e.o.p. 10^{-4}	Self	.
SW 623	<i>i</i> :-	e.o.p. low	<i>S. typhimurium</i> LT 2	+
SW 940	<i>a</i> :-	e.o.p. low	<i>S. sendai</i> SW 771	.
SL 117	<i>r</i> :-	e.o.p. low	<i>S. heidelberg</i> SL 142	+
SW 633	<i>l</i> , <i>z</i> :-	e.o.p. low	<i>S. paratyphi B</i> SW 546†	+
SL 163	<i>c</i> :-	e.o.p. low	<i>S. altendorf</i> SL 137	-
SL 159	<i>e</i> , <i>h</i> :-	-	<i>S. chester</i> SL 139	-
SL 165	<i>e</i> , <i>h</i> :-	-	<i>S. kaapstad</i> SL 136	-
SL 116	<i>g</i> , <i>p</i> :-	-	<i>S. dublin</i> SW 553 (O)	-
SW 679	<i>g</i> , <i>m</i> :-	-	<i>S. enteritidis</i> SW 764	-
<i>S. typhimurium</i>				
LT 2	<i>i</i> : <i>l</i> , <i>z</i>	+	.	.
Derivatives				
SW 698	<i>i</i> : <i>e</i> , <i>n</i> , <i>x</i>	+	<i>S. abony</i> SW 803	-
SW 699	<i>b</i> : <i>l</i> , <i>z</i>	+	<i>S. abony</i> SW 803	-
SL 141	<i>b</i> : <i>e</i> , <i>n</i> , <i>x</i>	+	<i>S. abony</i> SW 803 (two exposures)	-

Table 2 (cont.)

Strain	Antigen	Sensitivity	Donor of antigen	Sensi- tivity of donor*
<i>S. typhimurium</i>				
LL 22	<i>i</i> : 1, 2	+	.	.
Derivatives				
SW 674	<i>g, p</i> : 1, 2	.	<i>S. dublin</i> SW 553 (O)	—
Phase 1	<i>g, p</i>	—	.	.
Phase 2	1, 2	+	.	.
<i>S. heidelberg</i>				
SL 28	O strain	(—)	.	.
Derivatives				
SL 142	<i>r</i> : 1, 2	+	Self	.
SL 118	<i>i</i> : 1, 2	+	<i>S. typhimurium</i> SL 55	—
SL 121	<i>b</i> : 1, 2	+	<i>S. paratyphi B</i> SW 609	(+)
SL 119	<i>g, p</i> : 1, 2	.	<i>S. dublin</i> SW 553 (O)	—
Phase 1	<i>g, p</i>	—	.	.
Phase 2	1, 2	+	.	.
<i>S. dublin</i>				
SW 553	O strain	(—)	.	.
Derivatives				
SL 149	<i>g, p</i> : —	—	Self	.
SL 120	<i>b</i> : —	(+)	<i>S. paratyphi B</i> SW 543 (O)	e.o.p. 10 ⁻⁴
SL 122	<i>r</i> : —	(+)	<i>S. heidelberg</i> SL 28 (O)	+
<i>S. moscow</i>				
NCTC 5768	<i>g, p</i> : —	—	.	.
NCTC 5768. <i>i</i>	<i>i</i> : —	+	<i>S. typhimurium</i> SL 375	+
<i>S. essen</i>				
NCTC 5723	<i>g, m</i> : —	—	.	.
NCTC 5723. <i>i</i>	<i>i</i> : —	+	<i>S. typhimurium</i> LT 2	+
<i>S. hato</i>				
NCTC 9899	<i>g, m, s</i> : —	—	.	.
SL 512. <i>i</i>	<i>i</i> : —	(+)	<i>S. typhimurium</i> SL 375	+
<i>S. enteritidis</i>				
SL 513	<i>g, m</i> : —	—	.	.
SL 513. <i>i</i>	<i>i</i> : —	(+)	<i>S. typhimurium</i> SL 375	+
<i>S. enteritidis</i>				
SL 433	<i>g, m</i> : —	—	.	.
SL 433. <i>i</i>	<i>i</i> : —	(+)	<i>S. typhimurium</i> LT 2	+
<i>S. enteritidis</i>				
SL 431	<i>g, m</i> : —	—	.	.
SL 431. <i>i</i>	<i>i</i> : —	—	<i>S. typhimurium</i> SL 375	+
<i>S. enteritidis</i>				
SL 432	<i>g, m</i> : —	—	.	.
SL 432. <i>i</i>	<i>i</i> : —	—	<i>S. typhimurium</i> SL 375	+

* Some donors were themselves nonmotile: this is indicated by (O) after the name of the strain and the sensitivity given is that of motile variants of the strain.

† SW 546 is a monophasic strain with 1, 2 as its phase 1 antigen (Lederberg & Edwards, 1953).

‡ Clear plaques (see text).

(2) The introduction of H antigen $g...$ into a sensitive strain such as *Salmonella typhi*, SY 79 or SW 537, or the monophasic *S. paratyphi B*, SW 543, made the strain totally resistant. When antigen g,p replaced the natural phase 1 antigens i or r in the sensitive diphasic strains *S. typhimurium*, LT 22, or *S. heidelberg*, SL 28, respectively, the strains became resistant in this phase, while remaining sensitive in phase 2 (with antigen 1,2). Six out of eight monophasic strains which naturally carried antigen $g...$ and were resistant to the phage, became sensitive, or slightly sensitive, when another H antigen was substituted. Thus the resistance of some, although not all, natural strains carrying antigen $g...$ is due to the presence of this antigen.

When antigen l,z_{13} was introduced into *Salmonella typhi* SW 537, concentrated phage produced only a small number of discrete plaques. When antigen e,h was introduced into *S. typhi* SY 79 or SW 537, the phage produced a small number of clear plaques with a much larger number of very indistinct plaques. The ratio of the former to the number of plaques produced on NCTC 5727 was about 10^{-6} , and that of the latter was about 10^{-3} – 10^{-4} . When antigen e,h was introduced into the less sensitive *S. paratyphi B* SW 543, it made it totally resistant.

(3) A suitable H antigen was not the only requirement for sensitivity, for the reaction of the strain from which the antigen was transduced did not influence the reaction of the recipient, except where certain antigens, such as $g...$, l,z_{13} , or e,h were concerned.

Host-range variants

Several instances have been given where ordinary stocks of the χ phage had a very low e.o.p. on certain strains (e.g. 10^{-4} – 10^{-7}) so that high concentrations of phage produced only relatively few plaques. The phage present in these plaques plated on the test strain concerned with almost maximum efficiency (e.o.p. 0.1–1). When the variants were again grown on *Salmonella abortus-equi* NCTC 5727, their behaviour divided them into two groups.

(1) The first group was that in which the e.o.p. on the test strain had again fallen to about the original value, so that the variant stocks had presumably shown only phenotypic variation and did not consist of mutants. The test strains concerned were:

Salmonella paratyphi A strains A 203; A. BA 528; NCTC 9542; SL 31 (with antigen i substituted for a); and SL 37 (with antigen r substituted for a); *S. paratyphi B* strain SW 543S; *S. harrisonburg* strain NCTC 8258.

(2) The second group consisted of those variants which were evidently host-range mutants since their stocks grown on *Salmonella abortus-equi* NCTC 5727 retained a high e.o.p. on the test strain. The bacterial strains concerned here were:

SW 537. l,z_{13} (*S. typhi* H 901 in which H antigen l,z_{13} had been substituted for d);

NCTC 8717 (*S. ness-ziona* 6, 7 : l,z_{13} : 1,5). This strain was found to have been in phase 1 (with antigen l,z_{13}) when it was tested and the phage mutant was selected;

SL 651 (*S. shanghai* 16 : l,v : 1,6). This strain was found to have been in phase 1 (with antigen l,v) when it was tested and the phage mutant was selected;

SL 507	(<i>S. typhi</i> SY 79 in which H antigen <i>e,h</i> had been substituted for <i>d</i>);
NCTC 8278	(<i>S. kasenyi</i> 38 : <i>e,h</i> : 1,5);
NCTC 7318	(Arizona O antigen 9 : H antigen 13, 15);
NCTC 9896	(<i>S. thomaspville</i> 3, 15, 34 : <i>y</i> : 1,5);
SW 964S	(<i>S. typhimurium</i> , the motile variant of a strain, SW 964, which was received in the O state);
SL 653	(<i>S. typhimurium</i> C 56 of Boyd, which carries phage A2d).

In tests with a limited number of strains, the mutants showed no loss of activity for strains sensitive to the wild-type phage; thus their host-range was enlarged rather than diminished. Their plaques on *S. abortus-equi* NCTC 5727 and other strains sensitive to the wild-type χ phage were similar in appearance to the plaques of the wild type phage.

Table 3. *Activities of the host-range mutants against strains on which mutants were isolated*

Strain	e.o.p. χ	e.o.p. mutant of χ selected on strains					
		537. <i>l,z</i> ₁₃	8717	651	507*	8278*	7318
SW 537. <i>l,z</i> ₁₃	10 ⁻⁶ -10 ⁻⁸	0.5-1	0.5-1	0.5-1	≤ χ	≤ χ	≤ χ
NCTC 8717, phase with <i>l,z</i> ₁₃	10 ⁻⁶ -10 ⁻⁷	0.5-1	0.5-1	0.5-1	≤ χ	≤ χ	≤ χ
SL 651, phase with <i>l,v</i>	10 ⁻⁶ -10 ⁻⁷	0.5-1	0.5-1	0.5-1	≤ χ	≤ χ	≤ χ
SL 507 (<i>e,h</i>) (or SL 506, or SW 668)	10 ⁻⁶ *	≤ χ	≤ χ	≤ χ	0.5	0.5-1	≤ χ
NCTC 8278, phase with <i>e,h</i>	10 ⁻⁶ -10 ⁻⁷	≤ χ	≤ χ	≤ χ	0.01	0.2-0.7	≤ χ
NCTC 7318 (13, 15)	10 ⁻⁷ -10 ⁻⁸	≤ χ	≤ χ	≤ χ	≤ χ	≤ χ	0.1-0.2

The phages were titrated by the modified Miles & Misra method, using preparations which had been grown on NCTC 5727 and whose titres on this strain were 10¹⁰-10¹¹/ml. In some cases, a preparation grown on the strain which had selected the mutant was also tested, with similar results.

* Distinct, clear plaques (see text).

≤ χ the e.o.p. was no greater than that of wild-type χ phage.

Strains carrying H antigen l. The results of titrating the first six mutants on the bacterial strains by which each was isolated are summarized in Table 3. The results with the first three suggested that these strains had each selected a phage mutant which had gained the ability to attack bacteria carrying the H antigenic component *l*. Accordingly, strains with H antigen *l,z*₁₃, *l,v* or *l,w*, which had already been tested in the general host-range tests, were retested after separation into their two phases. Strains NCTC 8717 and SL 651, and all the strains which had appeared as fully sensitive were retested, together with most of the strains which had given a (+) reaction and a few of those which had appeared as resistant. Cultures in each phase were tested with the wild-type phage, and cultures, in the phase exhibiting antigen *l*... were tested with the mutant isolated from SW 537.*l,z*₁₃ and grown on NCTC 5727; the results are shown in Table 4, Section A. A few strains were also tested with the mutants isolated from NCTC 8717 or SL 651, with similar results. The results supported the conclusion that the wild-type phage was unable to attack bacteria with H antigen *l*..., but could mutate to a form able to do so.

Strains carrying H antigen e,h. When the wild-type χ phage was plated with strains SL 506, SL 507 or SW 668 (*Salmonella typhi* in which antigen *e,h* had been substituted for *d*), very small indistinct plaques could occasionally be seen with the higher phage dilutions, the e.o.p. being about 10^{-3} . The lower dilutions produced partial clearing in which there were discrete clear plaques; the ratio of these to the total number of plaques which the preparation produced on NCTC 5727 (or on the same strains of *S. typhi* carrying antigen *d*) varied between 10^{-6} and 10^{-7} . The phage isolated from these plaques, or from plaques similarly produced on strain NCTC 8278 (*S. kasenyi* 38 : *e,h* : 1,5) by the lower phage dilutions, gave clear plaques with high e.o.p. on the three *S. typhi* derivatives with antigen *e,h*, and also plated with high efficiency on strain NCTC 8278. Table 4, Section B shows the results when NCTC 8278 and 4 other strains with antigen *e,h* were tested in each phase with wild-type χ phage, and in the phase exhibiting *e,h* with the phage mutants isolated from SL 507 and NCTC 8278: these appear to be mutants which have gained activity for bacteria with H antigen *e,h*. The two mutants were not identical, for the one from NCTC 8278 plated with higher efficiency on these strains than the one from SL 507.

Table 4. *Sensitivity tests*

A. *Tests on naturally occurring strains carrying H antigens l,v, l,w, or l,z₁₃ in one phase*

	No. of strains	Reaction to χ		Reaction of cultures in phase with <i>l...</i> to mutant selected by SW 537. <i>l,z₁₃</i>
		Cultures in phase with antigen <i>l...</i>	Cultures in other phase	
Strains with <i>l,z₁₃</i> as one antigen	1	Plaques	+	+
	1	—	(+)	+
	3	—	(+)	(+)
	2	—	—	—
Strains with <i>l,v</i> as one antigen	2	Plaques	+	+
	2	(+)	+	+
	4	—	+	+
	2	—	(+)	(+)
	3	—	—	—
Strains with <i>l,w</i> as one antigen	1	Plaques	+	+
	6	—	+	+
	2	—	—	—

B. *Tests on naturally occurring strains carrying H antigen e,h in one phase*

	No. of strains	Reaction to χ		Reaction of cultures in phase with <i>e,h</i> to mutants selected by NCTC 8278 or SL 507
		Cultures in phase with antigen <i>e,h</i>	Cultures in other phase	
Strains with <i>e,h</i> as one antigen	1	Plaques	+	+
	2	—	+	+
	2	—	(+)	(+)

Arizona strain NCTC 7318. Arizona NCTC 7318 has H antigen 13, 15; since Arizona H antigen 13 closely resembles Salmonella H antigen *g* (Edwards & Ewing, 1955; Edwards *et al.* 1959), it seems likely that NCTC 7318 is resistant because it carries H antigen 13, and that the mutant isolated on it is one which has gained activity for bacteria with this antigen. No other strains with antigen 13, 15 were available; but, on 2 of the 4 Arizona strains with antigen 13, 14 which were resistant to the wild-type phage, the mutant from NCTC 7318 had some activity in that concentrated phage gave some degree of clearing, although the dilutions produced no plaques. A number of salmonella strains with antigen *g*... were tested with the mutant from NCTC 7318 but all were resistant; these included the artificial strains SL 119 and SW 674 in the phase exhibiting antigen *g,p* (which were sensitive in the other phase), and strains NCTC 5723 and NCTC 5768 (which became sensitive when antigen *i* was substituted for *g*...). Thus, if the mutant isolated on NCTC 7318 is one which has gained activity for bacteria with Arizona antigen 13, it can evidently distinguish between this antigen and the serologically related Salmonella antigen *g*. None of the other host-range mutants attacked bacteria with H antigen *g*.

Table 5. *Association between H antigenic type and reaction to the χ phage*

Antigen	Associated with	Evidence	
		Natural strains	Synthetic strains
<i>a</i>	Sensitivity	<i>S. abortus-equi</i> NCTC 5727 sensitive in phase 1 4 <i>S. paratyphi A</i> strains sensitive	<i>S. typhi</i> SL 508 sensitive
<i>b</i>	Sensitivity	3 strains <i>S. paratyphi B</i> sensitive in phase 1	<i>S. typhi</i> SL 502 sensitive <i>S. typhimurium</i> SW 699 sensitive in phase 1
<i>c</i>	Sensitivity	3 diphasic strains sensitive in phase 1	<i>S. typhi</i> SL 505 and SW 902 sensitive
<i>d</i>	Sensitivity	Most <i>S. typhi</i> strains sensitive	.
<i>i</i>	Sensitivity	<i>S. typhimurium</i> 1 monophasic strain and 2 diphasic strains sensitive in phase 1	<i>S. typhi</i> SW 520 and SW 569 sensitive
<i>r</i>	Sensitivity	2 diphasic strains sensitive in phase 1	<i>S. typhi</i> SL 504 sensitive
<i>z</i>	Sensitivity	2 diphasic strains sensitive in phase 1	<i>S. typhi</i> SW 537. <i>z</i> sensitive
<i>z</i> ₁₀	Sensitivity	2 diphasic strains sensitive in phase 1	<i>S. typhi</i> SW 537. <i>z</i> ₁₀ sensitive
<i>z</i> ₃₆	Sensitivity	1 monophasic strain sensitive	.
<i>z</i> ₄ , <i>z</i> ₂₃	Sensitivity	5 monophasic strains sensitive	.
<i>z</i> ₄ , <i>z</i> ₂₄	Sensitivity	2 monophasic strains sensitive	.
<i>e,n,x</i>	Sensitivity	4 <i>S. abortus-equi</i> strains sensitive	.

Table 5 (cont.)

Antigen	Associated with	Evidence	
		Natural strains	Synthetic strains
1,2	Sensitivity	5 monophasic strains sensitive	<i>S. typhi</i> SL 509 sensitive
1,5	Sensitivity	2 monophasic strains sensitive	.
1,7	Sensitivity	1 diphasic strain sensitive in phase 2	<i>S. abortus-equi</i> NCTC 5727.1,7 sensitive
z_6	Probably sensitivity	4 diphasic strains sensitive in phase 2	.
e, n, z_{15}	Probably sensitivity	3 diphasic strains sensitive in phase 2	.
1,6	Probably sensitivity	2 diphasic strains sensitive in phase 2	.
k	Probably sensitivity	2 diphasic strains sensitive in phase 1	.
l, z_{13}	Sensitivity to specific $l...$ variant	See Tables 3 and 4	.
l, v	Sensitivity to specific $l...$ variant		
l, w	Sensitivity to specific $l...$ variant		
e, h	Sensitivity to specific e, h variant		
y	Sensitivity to variant	3/4 diphasic strains sensitive to variant in each phase, but sensitive to wild-type χ only when not exhibiting y	.
z_{29}	.	2/9 monophasic strains (+) reaction	.
z_{38}	.	2/4 monophasic strains (+) reaction	.
$g...$	Resistance	0/50 monophasic strains sensitive. (1 slightly sensitive strain with m, t) 3 diphasic strains in $g...$ phase while sensitive in alternative phase	<i>S. typhi</i> SL 503, SL 510, SL 511, SW 667 resistant. SW 674, SL 119 resistant in phase with $g...$ and sensitive in alternative phase. When i was substituted for $g...$ naturally present, 6/8 strains became sensitive

The implications of the results with the last three strains are not clear. In the case of NCTC 9896 (*Salmonella thomasville* 3, 15, 34: $y: 1,5$), the H antigen appeared to be concerned, for this strain reacted differently in each phase, and the host-range of a phage mutant which it selected included (among other bacteria not sensitive to the wild-type phage) bacteria with H antigen y . When four strains with antigen y were tested, none was sensitive to the wild-type phage in the phase which exhibited it, but three were fully sensitive in the other phase; these three strains in the phase with y were sensitive to the mutant selected by NCTC 9896.

Strains SL 653 and SW 964S carry H antigens $i: 1,2$ which are associated with

sensitivity to the wild-type phage in other strains; cultures in each phase reacted in the same way, and the phage mutant which each strain selected had gained activity only against that strain itself.

Table 5 summarizes the results of testing the different H antigens. The antigens which occur naturally only in diphasic serotypes (those of Section 3 of Table 1) were tested in so far as possible after transduction to fully sensitive strains of *Salmonella typhi* (monophasic in phase 1), or *S. abortus-equi* (monophasic in phase 2). They were also tested by selecting predominantly monophasic clones from diphasic strains.

Association of sensitivity and motility

The association of sensitivity to the χ phage with the presence of flagella was investigated with (1) naturally occurring non-motile strains and with (2) phage-resistant variants selected from sensitive populations by the χ phage.

Naturally occurring non-motile strains. These comprised 62 strains of various serotypes. Motile derivatives of 45 of them were available and consisted either of spontaneous mutants or of derivatives obtained by transduction. The results are given in Table 6. All the non-motile strains were phage resistant and most of the motile derivatives were sensitive, with the consistent exception of strains which turned out to have H antigen g.... It will be noticed that the non-flagellated strains were not the only resistant ones; all of 10 paralysed strains were also resistant. The motile derivatives of these paralysed strains were sensitive. Table 6 therefore reveals a further condition determining sensitivity to the χ phage, namely, that the flagella must be active. This is well illustrated with the O strain of *Salmonella typhimurium*, SW 573, where mutation leading to flagella production (SL 43) was not enough for sensitivity; this only appeared when with further mutation the strain became motile (SL 43 swarm).

SJ 30, the 'curly' mutant of NCTC 5727 with short wavelength flagella, showed as marked clearing and as many plaques as NCTC 5727 itself. This variant was not truly paralysed for, although it did not exhibit translational motility, it did show very active jerking movement.

Experiments with Salmonella abortus-equi. The number of flagella formed by freshly isolated strains of this species, and hence their motility, depend on the pH of the medium; many flagella are formed at pH 6.5 and few at pH 8 (Kato, 1954). Three freshly isolated strains obtained by Dr B. A. D. Stocker from Dr Kato were tested and this observation was confirmed. Also, the strains were found to be sensitive to the χ phage on agar of pH 6.5 and resistant on agar of pH 8.0. Variants of these strains obtained by passage through semi-solid motility medium of pH 7.4 were as sensitive at pH 8.0 as at the lower pH.

Salmonella typhi: effect of the Vi antigen

Previous authors noted that Vi-positive strains showed weaker motility and lower sensitivity to the χ phage than Vi-negative strains. Broth cultures of all the Vi-positive strains used here contained over 50% motile bacteria and thus none was carrying sufficient Vi antigen to interfere seriously with its motility in broth. Nevertheless, 37 of 104 Vi-positive strains did not show maximum sensitivity to the χ phage, and in 21 of these, loss of the Vi antigen resulted in an increase of sensitivity.

Table 6. Action of the χ phage on non-motile *Salmonella* strains and their motile derivatives

Strain	Nonmotile strain		Motile derivative*		
	O or paralysed	Sensitivity	Strain	Antigen	Sensitivity
<i>S. typhi</i>					
SL 100	O	—	SL 100 S	<i>d</i> : —	+
SL 101	O	—	.	.	.
SL 102	O	—	.	.	.
SL 77 (0901)	O	—	SL 77 S	<i>d</i> : —	+
SL 436	O	—	SL 436 T	<i>d</i> : —	+
A C 6 6225	O	—	.	.	.
AT 820	O	—	AT 820 T	<i>d</i> : —	+
SL 232	Par. <i>d</i>	—	SL 232 S	<i>d</i> : —	+
<i>S. enteritidis</i>					
SW 971	O	—	SW 971 T	<i>g, m</i> : —	—
NCTC 203	O	—	.	.	.
NCTC 3045	O	—	.	.	.
NCTC 6676	O	—	NCTC 6676 S	<i>g, m</i> : —	—
<i>S. dublin</i>					
SW 553	O	—	SL 146 (S)	<i>g, p</i> : —	—
SL 76	O	—	NCTC 5766 (S)	<i>g, p</i> : —	—
SL 435	O	—	SL 435 T	<i>g, p</i> : —	—
Group D, unknown sp.					
SW 970	O	—	.	.	.
SW 972	O	—	.	.	.
<i>S. paratyphi B</i>					
SW 543	O	—	SW 543 S	<i>b</i> : —	low e.o.p.
SW 908	O	—	SW 908 S	<i>b</i> : 1, 2	+
SW 966	O	—	SW 966 T	<i>b</i> : 1, 2	(+)
SL 51	O	—	SL 51 T	<i>b</i> : 1, 2	+
A U 2	O	—	.	.	.
A U 15	O	—	A U 15 B (T)	<i>b</i> : 1, 2	+
A U 26	O	—	A U 26 M (T)	<i>b</i> : 1, 2	+
SR 107	O	—	SR 107 T	<i>b</i> : (1, 2)	(+)
SL 368	Par. <i>b</i> : 1, 2	—	SL 368 T	<i>b</i> : (1, 2)	(+)
SW 906	Par. <i>b</i> : 1, 2	—	SW 906 S	<i>b</i> : 1, 2	+
<i>S. typhimurium</i>					
SW 541	O	—	SW 541 T	<i>i</i> : 1, 2	—
SW 544	O	—	SW 544 S	<i>i</i> : 1, 2	+
SW 545	O	—	SL 89 (S)	<i>i</i> : 1, 2	+
SW 549	O	—	SW 594 (S)	<i>i</i> : 1, 2	+
SW 964	O	—	SW 964 S	<i>i</i> : (1, 2)	+
SW 965	O	—	SW 965 S	<i>i</i> : (1, 2)	+
SL 15	O	—	SL 15 T	<i>i</i> : (1, 2)	+
A U 14	O	—	A U 14B (T)	<i>i</i> : 1, 2	+
A U 20	O	—	A U 20M (T)	<i>i</i> : 1, 2	+
SL 56	O	—	SL 61 (S)	<i>i</i> : 1, 2	—
SW 573†	O	—	.	.	.
SL 43†	Paralysed from SW 573	—	SL 43 S	<i>i</i> : 1, 2	+
SL 499	Par. <i>i</i> : 1, 2	—	SL 499 S	<i>i</i> : 1, 2	+
SW 1153	Par. <i>i</i> : 1, 2	—	SW 1153 S	<i>i</i> : 1, 2	+
SW 578	Par. <i>i</i> : 1, 2	—	SW 582 (S)	<i>i</i> : 1, 2	+
SW 580	Par. <i>i</i> : 1, 2	—	SW 583 (S)	<i>i</i> : 1, 2	+
SJ 60	Par. <i>i</i> : 1, 2	—	SJ 60 S	<i>i</i> : 1, 2	+

Table 6 (cont.)

Strain	Nonmotile strain		Strain	Motile derivative*	
	O or paralysed	Sensitivity		Antigen	Sensitivity
<i>S. heidelberg</i>					
AU 1	O	—	AU 1 T	$r: 1, 2$	(+)
AU 21	O	—	AU 21 M (T)	$r: 1, 2$	—
SL 28	O	—	SL 142 (S)	$r: 1, 2$	+
<i>S. paratyphi A</i>					
A 17689	O	—	.	.	.
SL 14	O	—	.	.	.
NCTC 13	O	—	NCTC 13 S	$a: -$	low e.o.p.
NCTC 8052	O	—	.	.	.
NCTC 8285	O	—	.	.	.
NCTC 8388	O	—	.	.	.
NCTC 8389	O	—	.	.	.
<i>S. paratyphi C</i>					
SL 236	O	—	SL 236 S	$c: 1, 5$	+
SL 437	O	—	SL 437 S	$c: 1, 5$	+
<i>S. cholerae-suis</i>					
NCTC 5735	O	—	NCTC 5735 S	$c: 1, 5$	+
Group C1, unknown sp.					
AU 23	O	—	.	.	.
Group C2, unknown sp.					
AU 25	O	—	.	.	.
<i>S. riogrande</i>					
NCTC 7399.3	Par. $b: 1, 5$	—	NCTC 7399.3	$b: (1, 5)$	+
<i>S. milwaukee</i>					
NCTC 9890	O	—	NCTC 9890 S	$f, g: -$	—
<i>S. abortus-equi</i>					
SJ 30	'Curly'	+	SJ 30 S	$-: e, n, x$	+
	No translational motility				
SL 220†	.	—	SL 223 (S)	$-: e, n, x$	+
SL 221†	.	—	SL 224 (S)	$-: e, n, x$	+
SL 222†	.	—	SL 225 (S)	$-: e, n, x$	+

* Motile derivatives are designated S or T depending on whether they were obtained as spontaneous mutants or by transduction.

Antigens given in brackets were probably present in the strain although not detected in the culture tested.

† SL 43 was isolated from the non-flagellated *S. typhimurium* strain SW 573 as a flagellated but non-motile (paralysed) mutant (Stocker, Zinder & Lederberg, 1953).

‡ SL 220, SL 221, and SL 222 were strains of *S. abortus-equi* from Dr Kato described in the text. They produced few flagella and only a small proportion of the bacteria were motile in media of pH 7.2.

Resistant variants selected by the χ phage. Most of the strains found to be sensitive in the host-range tests were grown overnight in broth with the χ phage and examined microscopically the next day to estimate the proportion of motile bacteria. The strains fell broadly into four classes as shown in Table 7: class 1, 0–0.1% motile; class 2, 5–10% motile; class 3, 30–50% motile (over 80% of bacteria in control cultures were motile) and class 4 showing no decrease in the percentage motile.

Most strains fell into class 1. The three species *Salmonella typhi*, *S. paratyphi B* and *S. typhimurium*, of which a large number of strains were tested, behaved

differently in that most of the *S. typhi* and *S. paratyphi B* strains were in class 1 while the *S. typhimurium* strains were more evenly distributed amongst classes 1-4. Three of the strains of *S. typhimurium* tested more than once gave different results on different occasions, while the strains of the other two species gave consistent results in repeated tests.

Table 7. *Reduction of motility in overnight broth cultures infected with the χ phage*

Species	Number of strains in motility class *			
	1	2	3	4
<i>S. typhi</i>	79	5	3	0
<i>S. paratyphi B</i>	37	0	1	0
<i>S. typhimurium</i>	12 (+3)	12	14 (+3)	9
<i>S. paratyphi A</i>	5	0	0	0
<i>Others</i>	35 (+2)	11	4 (+1)	6 (+1)

* See text.

It was difficult to isolate stable phage-resistant mutants from most strains. The present experiments confirmed the finding of Rakieten & Bornstein (1941) that cultures which remained non-motile so long as phage was present could give rise to subcultures which regained motility when freed from the phage. This could be explained if all the genotypically motile bacteria present did not actually exhibit motility, and if phenotypic nonmotility was enough to make them resistant to the phage. Genotypically homogeneous salmonella strains have been studied in which not every bacterium is motile, but in which the proportion of motile organisms is a genetic characteristic of the strain (Quadling & Stocker, 1957). The phage might eliminate the motile bacteria, leaving those which were nonmotile either in genotype (none or a small minority) or merely in phenotype (the majority). Those that were merely phenotypically non-motile would give motile descendants. Some of the nonmotile bacteria in the culture may have been paralysed by the χ phage itself, as will be described later; but such bacteria might not give rise to colonies.

Table 8 shows the stable resistant variants that were obtained after attempts to isolate six variants from each of 26 strains. Some of these were flagellated but paralysed, again showing that flagellar inactivity leads to resistance. The resistant variants appeared to be of three types: non-flagellated; paralysed; slightly motile, i.e. broth cultures contained 25% or less of motile organisms often showing slow or irregular movement. As might be expected, variants belonging to the third class were not completely resistant; the most concentrated phage suspension often produced slight (+) clearing. Variants of this kind were chiefly found in strains of *Salmonella abortus-equi* but this was not due to reversion to the state described by Kato (1954) for freshly isolated strains, since these variants were not more motile, or more χ phage sensitive, at pH 6.5 than at pH 8.0. Examination of films from these cultures stained by Leifson's (1951) method showed that the mean number of flagella/bacterium was much lower than in their motile, phage-sensitive derivatives.

All the motile mutants of the resistant variants regained phage sensitivity.

Table 8. *Stable resistant variants selected by the χ phage from sensitive strains*

Strain	Stable resistants obtained:				Swarms obtained*
	Total	Nature			
		O	Paralysed	Slightly motile	
<i>S. typhi</i>					
A. A Cr.	4	4	0	0	O
A. C8 6608	4	3	1	0	1 (from O)
A. E4 5839	2	1	1	0	O
A. D1 5434	0
A. L2 131	0
SW 537 (H901)	7	4	3	0	4 (from O) 3 (from par)
SW 540	5	5	0	0	2 (from O)
<i>S. paratyphi</i> B					
A. 1927	3	3	0	0	2 (from O)
A. 3a 1	1	1	0	0	O
A. 1815	0
A. 1249	0
A. 1910	0
A. Workshop	0
SW 546 (1, 2 : -)†	1	1	0	0	1 (from O)
<i>S. typhimurium</i>					
SL 396	6	6	0	0	3 (from O)
LT 7	4	4	0	0	O
LT 2†	3	1	2	0	1 (from O) 1 (from par)
SW 593	6	6	0	0	5 (from O)
A. M4618	6	6	0	0	4 (from O)
A. U20M	6	6	0	0	3 (from O)
SL 394	0
SL 656	0
<i>S. abortus-equi</i>					
NCTC 5727	11	6	0	5	4 (from sl. mot.)
SL 224	6	4	0	2	2 (from sl. mot.)
<i>S. stanley</i>					
SW 536†	1	1	0	0	1 (from O)
<i>S. cholerae-suis</i>					
NCTC 5737	1	1	0	0	O
NCTC 5738	0
<i>S. memphis</i>					
NCTC 7402	4	3	0	1	2 (from O) 1 (from sl. mot.)
<i>S. riogrande</i>					
NCTC 7399	4	3	1	0	2 (from O) 1 (from par)

* All the swarms obtained from O, paralysed or slightly motile variants were highly motile and χ phage-sensitive.

† Resistant variants obtained from the collection of the Guinness-Lister Unit.

Adsorption experiments

The χ phage adsorbed slowly to many strains that were sensitive, there being no significant decrease in titre of free phage after 10–20 min. contact with the bacteria at concentrations of $2-5 \times 10^8$ organisms/ml. (Table 9). Thus, it was difficult to attribute the resistance of any given resistant strain to failure of adsorption. In

Table 9. Adsorption of the χ phage to naturally occurring motile strains

Species	Sensitivity	Number of strains with which phage in supernatant was		
		reduced to		not detectably reduced
		< 20 %	20–60 %	
<i>S. typhi</i>	+	6	2	0
	+(shallow plaques)	1	1	0
	(+)	1	1	1
	—	1	0	1
<i>S. abortus-equi</i>	+	2	0	0
<i>S. paratyphi B</i>	+	2	0	0
	+(shallow plaques)	1	0	0
	(+)	0	1	0
<i>S. typhimurium</i>	+	0	2	0
	+(shallow plaques)	0	0	6
	(+)	0	0	1
	—	0	0	1
<i>S. stanley</i>	+	1	0	0
	—	1	0	0
<i>S. paratyphi A</i>	—	1	0	0
Others, with H antigens other than <i>g...</i>	+	1	0	0
	+(shallow plaques)	1	2	8
	(+)	2	4	1
	—	1	1	5
With antigen <i>g...</i>	—	0	0	2

examining non-motile strains (either O or paralysed) only the results with strains whose sensitive motile variants absorbed the phage rapidly were considered to have any significance (Tables 10, 11) and the same considerations applied when the effects of different flagellar antigens were compared (Table 12).

A marked contrast was seen between:

(1) adsorption to naturally occurring non-motile strains and to their motile derivatives (Table 10);

(2) adsorption to non-motile or poorly motile derivatives isolated by the χ phage from naturally motile strains, and to motile forms of these strains (Table 11);

(3) adsorption to derivatives of a single strain carrying either an H antigen which prevents infection and of an H antigen which allows infection (Table 12). One of these strains, SW 674, gave very striking results since it absorbed rapidly in phase 2, with antigen 1,2, but not in phase 1, with antigen *g.p.* Only the host-range mutant to which the bacteria were sensitive, not the wild-type phage or other host-range mutants, adsorbed perceptibly to bacteria with H antigens *l...*, *e,h* or Arizona 13 (Table 12).

As shown in Tables 10 and 11, no detectable adsorption occurred to any of the non-flagellated strains, but two paralysed strains (SL 232 and SW 537.1a), and perhaps a third (SL 478) did produce a significant drop in the titre of free phage. With the paralysed strains of *Salmonella typhimurium* SL 43 and *S. riogrande* NCTC 7399.3, no antibody-resistant phage could be detected, and in an experiment with SL 232 in which 65 % of the phage was sedimented with the bacteria, only 0.08 % of the attached phage gave rise to infective centres after passage through antiserum. Thus, even if the phage does attach to a paralysed bacterium, infection is very unlikely to follow.

Table 10. *Adsorption to non-motile strains and their motile variants*

Strain	State of the organisms	Sensitivity	Phage remaining in supernatant %
<i>S. typhi</i>			
SL 77	Non-flagellated	—	NL
SL 77 swarm	90 % motile	+	10.5
SL 100	Non-flagellated	—	NL
SL 100 swarm	95 % motile	+	16.6
SL 232	Paralysed	—	63, 85
SL 232 swarm	60 % motile (slow)	+	30, 10
<i>S. paratyphi B</i>			
SW 906	Paralysed	—	NL
SW 906 swarm	95 % motile	+	16.5
<i>S. typhimurium</i>			
SW 573	Non-flagellated	—	NL
SI 43	Paralysed	—	NL
SL 43 swarm	90 % motile	+	40
LT 2	80 % motile	+	33
SL 499	Paralysed	—	NL
SL 499 swarm	90 % motile	+	48
SW 1153	Paralysed	—	NL
SW 1153 swarm	70 % motile	+	48
<i>S. riogrande</i>			
NCTC 7399	60 % motile	+	17.5
NCTC 7399.3	Paralysed	—	NL
NCTC 7399.3 swarm	90 % motile	+	12
<i>S. abortus-equi</i>			
SL 220	20 % motile, poorly flagellated	—	NL
SL 224	98 % motile	+	6
NCTC 5727	95 % motile	+	7.5
SJ 80	'Curly' flagella; rotating movement	+	NL

The adsorption mixtures were held at 37° for 10–20 min. before centrifugation.
 NL = no detectable loss of phage from the supernatant.

Resistance of a strain was not in all cases due to failure of adsorption. Four strains which appeared resistant in the usual sensitivity tests on agar, nevertheless, rapidly absorbed the phage. Two of these strains, *Salmonella stanley* NCTC 92 and *S. typhi* A D4 T 107, were also tested in broth; the results showed that although 85–95% of the phage had adsorbed, only 0.4% in the case of NCTC 92 and 3% in the case of A D4 T 107 gave plaques after passage through antiserum.

Association of flagella and adsorption

The effect of inhibiting flagella formation and of removal of flagella. Salmonella abortus-equi NCTC 5727, in common with other salmonellas, does not form flagella when grown at 44°. At 44° this strain grew more slowly than at 37°, reached a lower final concentration after overnight incubation and produced many short filaments.

Adsorption was measured at 44° and at 37° to cultures grown at each of these temperatures. No translational movement was seen in the culture grown at 44° although 1 % of the organisms were rotating, and there was no detectable adsorption

Table 11. *Adsorption to sensitive strains and χ -selected variants*

Strain	State of the organisms	Sensitivity	Phage remaining in supernatant (%)
<i>Salm. typhi</i>			
SW 537 (H901)	95 % motile	+	8.5
SW 537/ χ	Non-flagellated	—	NL
SW 537/ χ swarm	90 % motile	+	23
SW 537.1a	Paralysed	—	44
SW 537.1a swarm	90 % motile	+	12
<i>S. riogrande</i>			
NCTC 7399	95 % motile	+	17.5
NCTC 7399.6a	Non-flagellated	—	NL
<i>S. memphis</i>			
NCTC 7402	90 % motile	+	50
NCTC 7402.3a	Non-flagellated	—	NL
<i>S. typhimurium</i>			
LT 2	95 % motile	+	33
SL 478	Paralysed	—	70
<i>S. abortus-equi</i>			
NCTC 5727	95 % motile	+	7.5
3 isolates	Non-flagellated	—	NL
3 isolates	< 2 % motile	—	NL
3 isolates	c. 40 % motile	(+)	30–70
SL 224	90 % motile	+	6
SL 224.5b	1 % motile	—	NL
SL 224.5a	50 % motile	(+)	27

The adsorption mixtures were held at 37° for 10–20 min. before centrifugation.

NL = no detectable loss of phage from the supernatant.

after 15 min. at either 37° or 44°. On the other hand, there was good adsorption to the culture grown at 37°, in which 90 % of the organisms were normally motile, 86 % of the phage being absorbed at 37° and 91 % at 44°.

Flagella can be detached from bacteria without killing the organisms, either by short exposure to acid, by rubbing cultures over stiff agar, or by treatment in a Blender (Stocker & Campbell, 1959). Any of these methods greatly reduced the adsorption to *Salmonella abortus-equi* NCTC 5727 (Table 13), the blender being least effective, probably because the method leaves short stumps of flagella on the bacteria (Stocker & Campbell, 1959). The presence of residual flagellar fragments was indicated here by observing rotational movement in the blended suspension; the acid-treated and the rubbed suspensions contained virtually no motile bacteria.

Regeneration of flagella. Actively growing bacteria largely regenerate their flagella within one doubling time after treatment in the blender (Stocker & Campbell, 1959). The rate of adsorption of the χ phage to *Salmonella abortus-equi* NCTC 5727 increased progressively, while the bacteria were incubated in broth after blending (Fig. 1), and electron microscopy showed that during this time the flagella increased from small stumps to their normal length and also became more numerous.

Table 12. *Adsorption of wild-type χ phage, and host-range variants to bacteria with different H antigens*

Strain	Wild-type phage		Host-range mutants		
	Sensitivity reaction	Phage remaining in supernatant (%)	Mutant selected by:	Sensitivity reaction	Phage remaining in supernatant (%)
NCTC 5723 (<i>g,m</i>)	—	NL	.	.	.
NCTC 5723. <i>i</i> (<i>i</i>)	+	35	.	.	.
SW 674 <i>g,p</i> phase	—	NL†	.	.	.
SW 674, 1,2 phase	+	4	.	.	.
SW 537	+	15	.	.	.
SW 569 (<i>i</i>)	+	15	.	.	.
SW 667 (<i>g,p</i>)	—	NL‡	.	.	.
SW 537. <i>l,z</i> ₁₃ (<i>l,z</i> ₁₃)	Low e.o.p.	NL	SW 537. <i>l,z</i> ₁₃	+	18
			NCTC 8717	+	14
			SL 651	+	7
			NCTC 8278	< χ	NL
			NCTC 7318	< χ	NL
SW 668 (<i>e,h</i>)	Low e.o.p.	NL	NCTC 8278	+	50
		NL*	NCTC 8278	.	28*
			SW 537. <i>l,z</i> ₁₃	≤ χ	NL
			NCTC 7318	≤ χ	NL
SL 651 <i>l,v</i> phase	Low e.o.p.	NL	SW 537. <i>l,z</i> ₁₃	+	18
			SL 651	+	40
<i>S. victoria</i> (1, 9, 12 : <i>l,w</i> : 1,5)					
E 504, <i>l,w</i> phase	—	NL	SW 537. <i>l,z</i> ₁₃	+	12
E 504, 1,5 phase	+	30	.	.	.
NCTC 7318	Low e.o.p.	NL	NCTC 7318	+	36
		NL*	NCTC 7318	.	10*
			SW 537. <i>l,z</i> ₁₃	≤ χ	NL

The absorption mixtures were held at 37° for 10–20 min. before centrifugation.

* Adsorption mixture incubated for 2 hr. in the presence of chloramphenicol before centrifugation. NL = no detectable loss of phage from the supernatant.

† 0.07 % was found to survive exposure to antiphage serum, which probably represents the proportion of bacteria in the opposite phase.

‡ About 0.005 % survived exposure to antiphage serum, which was no more than in a control suspension without bacteria.

Tests for adsorption of phage to detached flagella

The results described so far suggest that the primary site of adsorption may be the flagella themselves. However, suspensions of flagella from sensitive strains detached from bacterial bodies neither inactivated the phage nor protected it from neutralization by antiphage serum (Table 14); nor did they promote infection of the

non-flagellated strain of *Salmonella typhi* O 901, whose flagellated and motile derivative, H901, is fully sensitive. Moreover, the phage did not appear to adhere to free flagella so far as could be seen in experiments in which it was added to free flagella which were later removed from suspension by adding H antibody and formalinized bacteria (to which the phage no longer adsorbs) carrying flagella of the same antigenic type. On centrifugation the bacteria were deposited, presumably taking with them the free flagella; the titre of free phage remained unchanged.

Table 13. Adsorption of the χ phage to bacteria whose flagella were removed by rubbing on stiff agar, by acid-treatment or by blending in an M.S.E. Blender

	Motility (%)	Viability (%)	Adsorbed phage (antibody- resistant) (%)	Unadsorbed phage (%)
Expt. 1				
Untreated	75	(a) } (b) } '100' (c) }	{ 90 91 87	14 18 15
Acid-treated (pH 2.6)	<0.1	(a) 25 (b) 6.3 (c) 12	0.4 2.2 .	85 100 95
Rubbed	<0.04	(a) } (b) } 100 (c) }	{ 0.29 0.1 .	88 96 100
Blended	<0.05 translational (some rotators)	(a) } (b) } 100 (c) }	{ 21 38 20	71 60 73
Expt. 2				
pH 2.2 $\frac{1}{2}$ min.	<0.1	60	0.3	.
pH 2.6 $\frac{1}{2}$ min.	<0.1	61	0.37	.
pH 3, 1 min.	<0.1	79	0.55	.
pH 7, 1 min.	80	'100'	75	.

S. abortus-equi NCTC 5727 was grown overnight on 1.25% (w/v) agar plates. For the 'rubbed' preparation, bacteria were transferred by wire loop to a 4% (w/v) agar plate and rubbed over it with a glass spreader for 3 min. They were then collected in distilled water and washed twice.

For the 'untreated', 'acid-treated' and 'blended' preparations, bacteria were gently soaked off in distilled water and washed once. The suspension was then divided into two parts; one part, which was to provide the untreated and acid-treated bacteria, was washed once again. The other part was blended for 3 min. and then washed once again. All the suspensions were adjusted to a total bacterial count of 5×10^{10} /ml., and dilutions in broth were examined for motility.

Acid treatment consisted in mixing 0.1 ml. of the previously untreated and well-washed suspension with 0.9 ml. of buffer pH 2.6 for 1 min. at 45°,* and then neutralizing with 9 ml. of either (a) buffer pH 7, (b) broth, or (c) broth containing 50 µg./ml. chloramphenicol. (Addition of either broth or buffer pH 7 raised the pH to about 6.) The neutralized suspensions were examined for motility. The 'untreated', 'rubbed' and 'blended' bacteria were similarly exposed to 45° for 1 min. using buffer pH 7; they were also tested after dilution into buffer pH 7, broth or broth with chloramphenicol.

Counts of viable bacteria were made, and adsorption of phage in each suspension was tested by mixing 7.5×10^7 phage particles with 6.5×10^9 bacteria, and counting: (1) the total number of plaques produced; and after 15 min. at 37° (2) the number of plaques after dilution through anti-phage serum (infected bacteria), and (3) the number of plaques produced by the supernatant after centrifugation.

In Expt. 2, the bacterial suspension, prepared as before, contained 3×10^{10} bacteria/ml. The acid was neutralized with buffer pH 7, and 3×10^9 /ml. bacteria were present in the adsorption mixture.

* The rate of flagellar lysis is temperature-dependent (Weibull & Tiselius, 1945; Duncan, 1935).

*Effect on adsorption of artificially paralyzing
Salmonella abortus-equi NCTC 5727*

It is clear from the preceding experiments that genetically paralysed bacteria are resistant to the χ phage and that this is associated with impaired adsorption of the phage. This observation raises the question as to whether a phage-sensitive motile strain would become resistant to the phage and absorb poorly if it were immobilized without loss of flagella. This can be done in several ways, for example, by thoroughly washing the bacteria (Stocker & Campbell, 1959). Experiments with *Salmonella abortus-equi* NCTC 5727 showed that bacteria grown in broth were still motile when resuspended in 10^{-4} M-phosphate buffer containing 10^{-3} M- CaCl_2 which

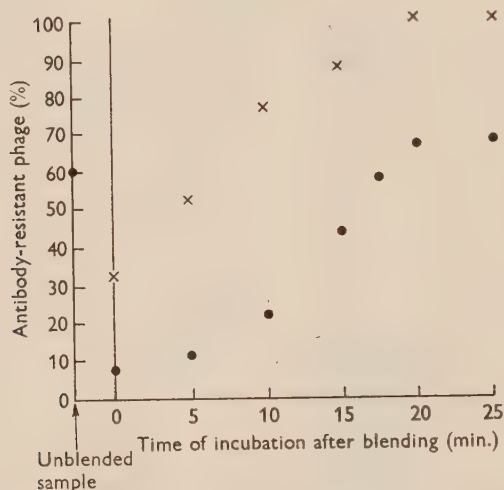


Fig. 1. Regeneration of flagella and adsorption. An actively growing culture of *Salmonella abortus-equi* NCTC 5727 was blended for 3 min. to detach the flagella and then incubated at 37° in a shallow layer of broth in a flask. Immediately after blending, and at 5, 10, 15, 17½, 20 and 25 min., samples were transferred to tubes containing 50 µg./ml. chloramphenicol (which arrests flagellar synthesis; Kerridge, 1959) and held at room temperature. The samples were examined for motility and total bacterial counts were made on the first and the last. *Sample taken immediately after blending*: total count, 2.7×10^8 /ml.; motility, 0.93 % transitional movement, 22% rotational movement.

Sample taken at 25 min.: total count, 4.6×10^8 /ml.; motility, 82 % translational movement. Samples taken at intermediate times showed progressively increasing proportions of motile bacteria; at first rotating bacteria predominated; at 15 min. 33 % of the bacteria showed translational movement which was rather slower than normal; and at 20 min. 60 % appeared to be fully motile.

When all the samples had been collected, phage was added to each to a concentration of 7.5×10^6 p.f.p./ml. The numbers of infected bacteria (●) 2 min. and (x) 20 min. later were measured by plating the mixtures after exposure to antiphage serum.

must have contained only about 1 % (v/v) broth, and the phage adsorbed normally. However, after washing 3–4 times in buffer, no bacteria showed translational motility and less than 5 % were rotating. Phage adsorption to such suspensions was greatly decreased (Table 15); it increased again when the bacteria regained motility, which they did immediately after either broth or histidine were added to the buffer suspension (Stocker & Campbell, 1959).

Similar results were obtained with bacteria immobilized by several unrelated agents such as chloral hydrate or Merthiolate (Eli Lilly & Co) (Robertis & Peluffo, 1951), 2,4-dinitrophenol, aureomycin or erythromycin (Table 14). 2,4-Dinitrophenol at $2.5 \times 10^{-3}M$ or more was needed; and its action was immediately

Table 14. Tests for adsorption of the χ phage to detached flagella

Mixtures		Total p.f.p.	Proportion of antibody- resistant phage (%)	Phage in supernatant
Expt. 1.				
Flagella - : 1,2. Phage	15 min., 37°	7.1×10^8	<0.05	.
Flagella i : 1,2. Phage	15 min., 37°	7.1×10^8	<0.05	.
Broth. Phage	15 min., 37°	6.7×10^8	<0.05	.
Expt. 2.				
Flagella - : 1,2. Phage	15 min., 37°	1.2×10^9	<0.09	.
Buffer. Phage	15 min., 37°	1.1×10^9	<0.09	.
Expt. 3.				
Flagella - : 1,2; O 901 2.5×10^8 /ml. Phage	20 min., 37°	.	<0.04	.
Flagella i : 1,2, O 901 2.5×10^8 /ml. Phage	20 min., 37°	.	<0.04	.
O 901 2.5×10^8 /ml. Phage	20 min., 37°	.	<0.04	.
Broth. Phage	20 min., 37°	7.9×10^8	<0.04	.
Expt. 4.				
Flagella - : 1,2. Phage	5 min., 37°	7.6×10^7	<0.013	7×10^7
After 5 min. formalinized. SW 1061 added		.	.	.
Flagella - : 1,2. Phage Formalinized SW 1061	10 min., 37°	8.7×10^7	<0.01	5.1×10^7
Formalinized SW 1061. Phage	10 min., 37°	6.2×10^7	<0.01	6.3×10^7
Broth. Phage	10 min., 37°	6.6×10^7	<0.01	5.7×10^7 *

In each individual experiment, the mixtures contained the same amount of phage, and after the time stated were assayed for total number of plaque-forming particles (p.f.p.) and by dilution through antiphage serum for antibody-resistant phage. Expt. 3 was made to see if the presence of flagella would promote infection of the non-flagellated strain of *S. typhi*, O 901. In expt. 4, a test was also directly made for physical adherence to flagella: formalinized bacteria of *S. typhimurium* SW 1061 (- : 1,2) were added to the mixtures either at once or after 5 min., and H antibody 1,2 was subsequently added to agglutinate the free flagella (Craigie, 1931) and, it is presumed, to cause their attachment to bacteria whose flagella were of the same antigenic type. After centrifugation, the supernatant was assayed for unattached phage.

* Here there were no bacteria or flagella present, and the mixture was not centrifuged; the figure given is the plaque count after addition of anti-1,2 serum, which did not affect the phage.

The flagellar suspension in Expt. 4 was used at a final dilution of 1/20, and a dilution of 1/2 in the first mixture before the addition of the formalinized bacteria. In Expts. 1-3 the suspensions were used at a dilution of 1/10, at which they showed a faint opalescence.

annulled on dilution to below $3 \times 10^{-4}M$. Chloral hydrate and Merthiolate have been reported to immobilize *Proteus vulgaris* reversibly, but in the present case chloral hydrate, and to a lesser extent, Merthiolate, at concentrations sufficient to immobilize, killed a considerable proportion of the bacteria. Aureomycin was more effective in immobilizing than erythromycin, but a high concentration of each was needed and both took about 30-40 min. at room temperature to produce their full

Table 15. *Adsorption to artificially paralysed NCTC 5727*

Bacteria treated with:	Motility	Phage in supernatant (%)	Motility restored by	Phage in supernatant (%)
Washing	0 % translational 1-5 % rotating	70-80	1/5-1/25 broth (motility 90-100 %) 1/75 broth (motility 5 % slow) 0.01 M histidine (motility 40 % slow + 10 % rotating) 0.05 M histidine (50 % slow)	6-15 50 34-47 40
Merthiolate				
9 × 10 ⁻³ M-3 × 10 ⁻⁸ M	1-10 %	NL	.	.
9.8 × 10 ⁻⁴ M	40 %	31	.	.
3.4 × 10 ⁻⁵ M	95 %	12	.	.
Chloral hydrate				
0.12 M	0	75	.	.
2,4-dinitrophenol				
2.5 × 10 ⁻³ M	0	80	Removal of drug (60	22
3.3 × 10 ⁻³ M	2 % rotating	76	80 % motility)	14
Aureomycin				
0.5 mg./ml.	(a) 40 min., < 1 % translational, 5 % rotating (b) 2 min., 90-98 % motile	60-100 10-20	.	.
Erythromycin				
0.5 mg./ml.	40 min., 2 % translational 5-50 % rotating	60	.	.
Terramycin				
0.5 mg./ml.	98 %	7	.	.
Streptomycin				
0.5 mg./ml.	98 %	6	.	.
Chloramphenicol				
0.5 mg./ml.	95-98 %	4-9	.	.

Adsorption was measured after 10 min. to late log or stationary phase bacteria at about 5×10^8 /ml., with a phage multiplicity of less than 1. In a few experiments with bacteria treated by washing, dinitrophenol or aureomycin, the adsorption mixtures were also assayed after passing through antiphage serum to measure the proportion of infected bacteria. This agreed in all cases with the estimates obtained from assay of the supernatant. In each experiment, control bacteria which had not been treated left 10 % or less of the phage in the supernatant.

Bactericidal effect of the drugs; survival after 15 min. exposure:

Merthiolate	9 × 10 ⁻³ M	10 %	Aureomycin	1 mg./ml.	50 %
	1.7 × 10 ⁻³ M	50 %	Erythromycin	1 mg./ml.	30 %
Chloral hydrate	0.12 M	< 0.1 %	Terramycin	1 mg./ml.	90 %
2,4-dinitrophenol	2.5 × 10 ⁻³ M	85 %	Streptomycin	1 mg./ml.	46 %

Merthiolate, 3×10^{-3} M for 15 min., inactivated 40 % of the phage. The phage was stable in the other drugs over the period of the experiments.

effect. Before it had affected the motility of the bacteria, aureomycin did not affect adsorption of the phage. The effect of aureomycin on motility may not have been reversible, but electron micrographs showed that the flagella were not destroyed. Terramycin, streptomycin and chloramphenicol did not affect motility or phage adsorption. It may be concluded from these experiments that bacteria which are merely phenotypically non-motile adsorb the χ phage more slowly, just as do genotypically paralysed strains.

Motility was irreversibly abolished by formaldehyde or by heating at 56° for 30 min.; no detectable adsorption occurred to cultures treated in either way. Neither treatment is supposed to alter the H antigen, but formaldehyde evidently denatures the flagellar protein (Astbury, Beighton & Weibull, 1955).

Electron microscopy

Electron micrographs of formalin-fixed, air-dried platinum + iridium-shadowed mixtures of *Salmonella abortus-equi* NCTC 5727 and the χ phage, which had been washed to remove the free phage, showed phage particles on the flagella, apparently attached by the tips of their tails (Pl. 2, figs. 3 and 4). The tails of some particles were curved as if they were genuinely attached in this way and had been bent while the specimens were prepared (Pl. 2, fig. 5). The bacteria could evidently absorb only a limited number of particles, as shown by estimates of the proportions of unadsorbed phage in an experiment where bacteria were mixed with varying concentrations of phage. The capacity appeared to be about 6 p.f.p.; if only about 1/5 of the total particles were plaque-formers, as the particle counts by fluorescence microscopy suggested, this would represent about 30 particles/bacterium, which was about the maximum number seen on electron microscopy.

It was impossible to say that there were no phage particles attached to the bodies of the bacteria, for phage particles were often seen near the body; but, as there was always a network of flagella in the region, they might as well have been attached to a flagellum as to the body itself. Control preparations of *Salmonella abortus-equi* NCTC 5727 with phage P22 (Pl. 2, fig. 6) showed many particles of this phage along the bodies of the bacteria, and none on the flagella, which strongly suggested that particles of the χ phage seen on the flagella were specifically attached. Attachment appeared to occur anywhere along the length of a flagellum, but groups of two, three, or more particles were commonly seen attached at or around a single point (Pl. 2, fig. 7). In contrast, phage stocks did not contain clumped particles except rarely, and then these were centred round a fragment of detached flagellum. The presence of these clusters suggests either that some portions of a flagellum favour phage attachment, or that the attachment of one particle encourages the attachment of others.

When the bacteria had been deflagellated in the blender before mixing with the χ phage, an occasional phage particle was seen on the remaining flagellar stumps; none was seen on the bodies of the bacteria. When the bacteria were blended after mixing with the phage, fewer phage particles were seen than with bacteria which had been blended before exposure to the phage; this suggests that the distal parts of flagella which are removed by blending can compete for adsorption of the phage with the proximal parts which survive blending.

The synthetic strain SW 674 (*g,p*: 1,2) was examined in both the phase

exhibiting 1,2, which was phage sensitive, and the phase with *g,p*, which was resistant. The flagella of the two phases did not differ in appearance, but large numbers of particles were seen on the flagella exhibiting antigen 1,2 while none was seen with those exhibiting antigen *g,p*. Analogous observations were made on the following strains in either their paralysed or their normally motile forms: *Salmonella typhimurium* SW 1153, a paralysed variant of strain LT2; *S. riogrande* NCTC 7399.3 (40 : *b* : 1,5); *S. typhi* SW 537.1a. The latter is the only one of these three paralysed strains to which any adsorption of the phage was detected and here electron microscopy showed an occasional particle attached to the flagella. Individual flagella appeared normal in bacteria immobilized by aureomycin although the flagella tended to form skeins. The numbers of particles seen on the flagella decreased only when the motility had fallen, 30–40 min. after exposure to the drug.

Immobilization and clumping

There are few published observations on the effect of phage infection on motility, but infection by most phages seems to leave motility unaffected until the latent period is advanced, or even until lysis (Murphy, 1957). *Salmonella abortus-equi* NCTC 5727 is certainly not immobilized for some time after adsorption of a clear-plaque mutant of phage P22, and the same is probably true of a motile strain of *Escherichia coli* B (Furness & Rowley, 1955) and phage T2. (Bacteria of this latter organism, unlike salmonellas, tend to stop swimming in preparations held between slide and coverslip; however, no difference could be observed between bacteria which had absorbed phage T2 and control preparations of uninfected bacteria.) On the other hand, immediately after mixing salmonella NCTC 5727 with the χ phage at sufficiently high multiplicity, the bacteria became immobile and formed clumps of 10–20 bacteria. The effect was most striking, and could be readily observed when a drop of high titre phage stock was placed on a slide at one edge of a coverslip, a drop of highly motile culture placed at the opposite edge and the two allowed to mix gradually. Where they met, rapidly swimming bacteria came to an abrupt halt and then often joined other already immobilized bacteria to form clumps. Agglutination of infected bacteria was reported by Beardsley (1960), but only began when new phage started to be released; a loss of motility which this author also mentioned evidently did not immediately follow on infection.

Immobilization of *Salmonella abortus-equi* NCTC 5727 by the χ phage could be prevented either by treating the phage stock with antiphage serum or by removing the phage particles by centrifugation. However, phage inactivated by ultraviolet radiation or by over-centrifugation appeared to immobilize as efficiently as infective phage, suggesting that the effect was due to adsorption of the phage, which need not be followed by multiplication.

When the phage was diluted, progressively fewer bacteria were immobilized. The process went rapidly to completion with *Salmonella abortus-equi* NCTC 5727; with a concentration of phage which left some motile bacteria, the proportion of these did not alter perceptibly after the first few minutes. Serial dilutions of several different phage preparations were tested for their immobilizing activity by estimating the proportions of bacteria which still showed translational motility. In each experiment the immobilizing activity decreased more rapidly with dilution of the phage than would be expected if one phage particle per bacterium were sufficient. The

possibility is not excluded that an individual flagellum might be inactivated by adsorption of a single particle.

Other sensitive strains besides *Salmonella abortus-equi* NCTC 5727 were also immobilized by the χ phage, but generally the process was slower, and there was less clumping of the immobilized bacteria. Among the strains that were tested was SW 674 which was rapidly immobilized in the phase with H antigen 1,2, but showed no loss of motility in the phase with *g,p*. Strains SW 537.*l,z*₁₃, NCTC 8717 exhibiting *l,z*₁₃, and SL 651 exhibiting *l,v*, were immobilized by the phage mutants which they had selected, but not by the wild-type phage or the other host-range mutants. Strain E 504 (*l,w* : 1,2), in the phase with 1,2 was immobilized by either wild-type phage or the mutant selected by SW 537.*l,z*₁₃, but in the phase with *l,w* it was immobilized only by the latter. Analogous results were obtained with strains SL 507 (*S. typhi* carrying *e,h*) and NCTC 8278 (*e,h* : 1,2) which was tested in each phase. Similarly, Arizona NCTC 7318 was slowly immobilized by the mutant which it had selected, but not by the wild-type phage or other mutants. Each of the host-range mutants rapidly immobilized *S. abortus-equi* NCTC 5727. Experiments were made with NCTC 5727 whose flagella had been removed by the blender. Here, since the bacteria were necessarily immobile, only clumping could have been observed. None occurred. Thus it appears that the clumping is a sequel to attachment of the phage to the flagella. Immobilization and clumping following phage adsorption was not accompanied by morphological changes in the flagella so far as could be seen by electron microscopy.

DISCUSSION

Previous authors reported that the χ phage attacked only flagellated bacteria, but it is now clear that flagella alone do not lead to susceptibility; these must be both active and of a correct antigenic type. They must also be present in suitable bacteria, for not all motile strains with the correct H antigens were sensitive. Resistance in the latter case might be due to inability of the phage to multiply in the bacteria (particularly with the few strains to which the phage readily adsorbed); but when resistance was associated with absence of suitable flagella, it was evidently due to failure of adsorption. The importance of the H antigen in the control of susceptibility immediately suggests that the phage adsorbs to the flagella themselves, since the H antigen is present only on the flagella and does not extend over the surface of the bacterial body (Craigie, 1931; Stocker & Campbell, 1959). Removal of the flagella from sensitive bacteria impaired adsorption. Direct evidence for attachment of phage particles to active flagella of correct antigenic type was provided by electron micrographs.

The importance of the bacterial strain itself in the control of sensitivity was most clearly seen when two strains carrying the same H antigen differed in their reactions, although the H antigenic determinant of the one had been received from the other by transduction, when the antigenic complex is known to be transferred unaltered (Lederberg & Edwards, 1953). Presumably, either the phage could not multiply in the resistant strain or the flagella of the two strains differed significantly in characteristics, other than the H antigen, which affected adsorption or initiation of infection. Transduction of the H antigenic determinant is unlikely to change all the

genes concerned with flagella (Stocker *et al.* 1953); hence, in a hybrid resulting from transduction of an H antigen, the other flagellar characters would probably be those of the recipient and not those of the donor strain.

The phage presumably failed to adsorb to isolated flagella for the same reason as it failed to adsorb to paralysed strains, namely, because the flagella were not functioning. Flagella such as are carried by salmonellas are helical in shape (Leifson, Carhart & Fulton, 1955); in living preparations of moving bacteria, they appear as rotating spirals (Reichert, 1909; Pijper, 1938; Weibull, 1950, 1951), and in fixed preparations they are flattened into a sinuous form. It is currently thought (Astbury *et al.* 1955) that motility as well as the helical shape of flagella result from the passage of a spiral wave of contraction down the flagellum, the helical line of contraction which is continuously moving along being due to the transmission of contractile pulses in subfibrils of which the flagellum is probably composed. The reflexions seen in X-ray analysis of flagellar preparations have been interpreted to indicate the presence of polypeptide chains in two different states of folding, and it is thought that the undulations leading to active flagellar movement are brought about by a rhythmical change of length between one configuration and the other. The two polypeptide chain configurations are always present simultaneously in preparations of flagella detached from the bacterial body, and these, in addition, retain their wave shape; thus it is supposed that flagella detached in the course of transmitting the wave of contraction remain fixed exactly as they were at the moment they were broken off (Astbury *et al.* 1955). Paralysed and motile bacteria are morphologically and serologically similar. The flagella of paralysed bacteria, although not moving, show no difference in wave form from the flagella of motile ones, and no difference in X-ray diffraction pattern (Beighton, Porter & Stocker, 1958). In particular, flagella isolated from paralysed bacteria also possess the features which indicate that the protein is present in two different configurations. Thus motile and paralysed flagella may both have similar helical lines of contraction, but in a paralysed flagellum this is static and not transmitted as a wave. In other words, isolated flagella and flagella of paralysed bacteria are thought to have the same array of features as active flagella, which consequently do not possess any unique structures that could be tentatively identified with the phage receptor. Two possible explanations for the need for active flagella are either that the phage receptor becomes unmasked during active movement, or that functioning of the flagella is required *per se* for adsorption either to occur, or to be irreversible and to lead to infection. The slight adsorption which was detected with a few paralysed strains, which was not followed by infection, may mean either that attachment can occur with decreased efficiency, perhaps transiently, to inactive flagella, or that the flagella of these particular strains had perhaps some slight activity short of that required for motility. The degree of activity associated with motility is evidently necessary for initiation of infection, possibly for the injection of the phage DNA (Hershey & Chase, 1952).

It was suggested by London (1958) that the site of adsorption of the χ phage to the bacterium may be at the basal granule of the flagellum. However, the present electron micrographs show phage particles attached along the length of the flagella, distally as well as proximally. In addition, comparisons of untreated and blended bacteria have shown that phage particles adsorbed distally can certainly infect the bacterium:

(1) When the flagella have been reduced to short stumps by blending, not only is the rate at which the phage particles attach to the bacteria decreased, but so is the rate at which the bacteria become infected (as measured by antibody-resistant infective centres) and this returns progressively to normal as the flagella regenerate (Fig. 1). The alternative explanation for this finding, namely, that the motility of the flagellated bacteria increases the chances of collision between the phage and the proximal part of the flagella, seems intuitively unlikely to account for the magnitude of the difference.

(2) If phage particles which have attached to distal parts of a flagellum fail to infect, then a phage preparation should show a higher titre when titrated by adsorption to blended bacteria than to bacteria with long flagella, provided a sufficiently long time is allowed for adsorption; no difference in titre is observed.

If, therefore, the primary site of phage adsorption can be at any point along the flagellum, the phage genome might reach the bacterial body by any of the following routes:

(a) by passage of the entire phage particle up the outside of the flagellum until it reaches the junction with the body and there injects its DNA. Although no precise estimates have been made, electron micrographs of samples of phage-bacterium mixtures, which had been fixed at different times after mixing, gave no indication of a drift of phage particles up the flagella towards the bacterial bodies;

(b) by adsorption of the phage particles to the bacterial body in the usual way, following some kind of 'activation' by contact with a flagellum. If this were so, then a phage particle after attachment to a flagellum belonging to one bacterium might be able to infect a different bacterium, but there was no indication that this could occur;

(c) by being injected into the flagellum at the point of initial attachment, and then travelling in the flagellum into the bacterial body. The pattern common to larger flagella and cilia is evidently a cylindrical arrangement of 9 subfibrils enclosing 2 more, and theoretically this allows the latter to roll round in the cylinder (Astbury *et al.* 1955). If bacterial flagella are structurally similar, the χ phage DNA might pass along the channels or potential channels that this arrangement might provide. Electron microscopy has sometimes suggested that bacterial flagella are made up of subfibrils (Starr & Williams, 1952; Labaw & Mosley, 1955) although in most electron micrographs, including the present ones, no fine structure can be seen.

With fully motile bacteria, the phage genome evidently reaches the bacterial body not long after adsorption, for the stages could not be separated by removing the flagella: when bacteria were mixed with phage for 1 min. (when 50 % of the phage had adsorbed) and then blended for 1.5 min., there was no decrease in the total number of plaque-forming entities (i.e. infected bacteria + free phage) nor in the number of infected bacteria alone (measured by the number of antibody-resistant infective centres sedimented by centrifugation at 1100 g.) Nor was there any increase in number of non-sedimentable p.f.p. as might occur if phage in a state able to infect new bacteria were detached with the flagella.

The sudden loss of active flagellar movement upon adsorption of phage seems to be an effect peculiar to the χ phage. In general, phage infection interferes only with bacterial syntheses, not with energy-yielding processes (Cohen, 1949) which, together with the fact that more than one particle of the χ phage per bacterium is needed, may imply that the immobilization observed here is due to a direct effect on the flagella.

This might be either an effect on the energy supply or on the structure of the flagella. Attachment of coliphage T2 results in activation and liberation of phage enzymes, notably the phosphatase (Dukes & Kozloff, 1959; Kozloff & Lute, 1959), and the cell-wall lytic enzyme (Weidel & Primosigh, 1958) which can cause gross morphological changes in the bacterium. For example, adsorption of coliphage T2 causes isolated bacterial cell walls to shrivel (Williams & Fraser, 1956). Thus, by analogy, adsorption of the χ phage to the flagellum might well cause gross changes in flagellar structure and immediate loss of activity. Such changes could also account for the agglutination that follows phage adsorption, since flagella (Scholtens, 1938) and their H antigens (Ogonuki, 1940) are known to influence the stability of flagellated organisms in suspension. Agglutination is not due to immobilization *per se* since genetically and artificially paralysed bacteria do not clump. It is interesting to note that two strains with abnormal 'curly' flagella, *Salmonella abortus-equi* SJ 30 and a similar variant of a *S. typhimurium* strain (Dr Iino, personal communication), agglutinate spontaneously whereas their derivatives with normal flagella are stable.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Particles of the χ phage negatively stained with phosphotungstic acid. $\times 163,000$.

Fig. 2. Particles of the χ phage negatively stained with phosphotungstic acid. Some collapsed heads can be seen. $\times 130,000$.

PLATE 2

Fig. 3. Particles of the χ phage on the flagella of NCTC 5727: fixed, shadowed preparation. $\times 24,300$.

Fig. 4. Particles of the χ phage on the flagella of NCTC 5727: fixed, shadowed preparation. $\times 7800$.

Fig. 5. Particles of the χ phage on a flagellum of NCTC 5727: the tail of one is bent (see text). Preparation negatively stained with phosphotungstic acid. $\times 270,000$.

Fig. 6. Particles of phage P22 surrounding the bacterial bodies of NCTC 5727: fixed, shadowed preparation. $\times 8000$.

Fig. 7. Cluster of particles of the χ phage (see text). $\times 8000$.

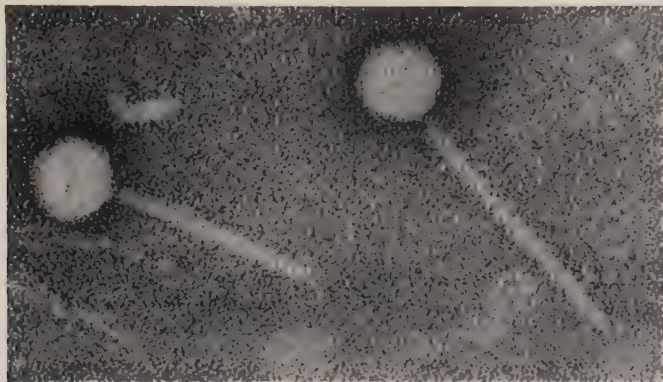


Fig. 1



Fig. 2

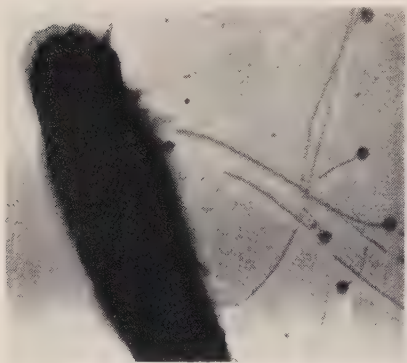


Fig. 3

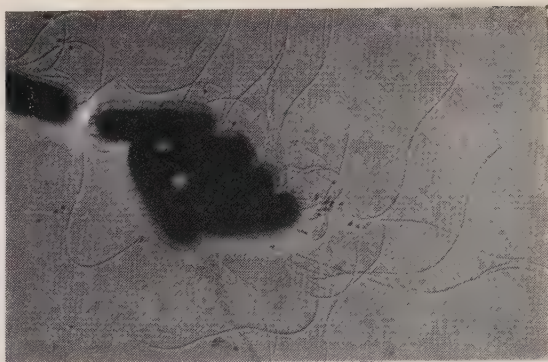


Fig. 4

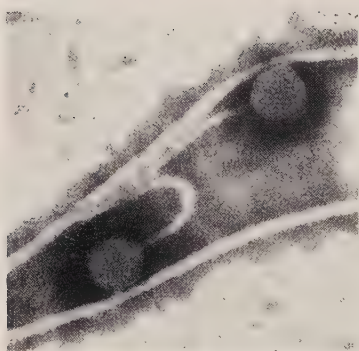


Fig. 5



Fig. 6

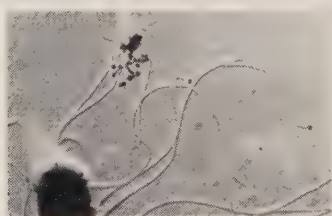


Fig. 7

Substances which Protect Lyophilized *Escherichia coli* against the Lethal Effect of Oxygen

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SUMMARY

The lethal effect of oxygen on *Escherichia coli* organisms freeze-dried from water may be partly abolished by the addition of certain compounds before lyophilization. Three groups of such protective compounds were found: (i) thiourea, some of its derivatives and analogues; (ii) sugars, especially monosaccharides and some of their derivatives; (iii) some simple inorganic salts. The protective action was not unequivocally correlated with reducing power. Certain reducing agents (e.g. sodium dithionite, cysteine, reduced glutathione) even enhanced the lethal effects of oxygen.

INTRODUCTION

It was shown previously (Lion & Bergmann, 1961) that *Escherichia coli* organisms lyophilized from distilled water, were rapidly inactivated by air and oxygen, even at low pressures. The usual freeze-drying procedure in which primary drying is followed successively by admission of dry air into the system, followed by secondary drying, cannot therefore be applied to organisms suspended in distilled water. It is possible, however, to preserve successfully the viability of the organisms under these circumstances when the lyophilization is carried out in the presence of a protecting medium such as that proposed by Naylor & Smith (1946). The identity of the substance or substances in this medium, which protect the dried bacteria against oxygen, has now been studied. The results, as well as experiments with other compounds likely to possess protective power, are reported in the present paper.

METHODS

The methods for growing the bacteria, the determination of viability, and the freeze-drying technique were described previously (Lion & Bergmann, 1961).

The bacteria were harvested and washed well in distilled water. To make up the final suspension, 0.2 ml. of a solution of the compound to be tested, at $5 \times$ the final concentration needed, was added to 0.8 ml. of the bacterial suspension in water, containing about 1.5×10^{11} bacteria. All the test solutions were sterilized by filtration.

Lyophilization was carried out at room temperature. At the end of the primary drying period, dried air was admitted to the system. The rubber tubes connecting the ampoules to the manifold were clamped off. The manifold was disconnected from the freeze-drying apparatus and transferred to a constant temperature incubator at 28°. The control ampoules to be kept under vacuum were clamped off before

air was admitted to the apparatus. Although flame sealing of ampoules under vacuum is much to be preferred, this is very difficult to carry out routinely on neutral glass ampoules, without constricting the neck. In practice, during 4 hr. (the duration of the experiments described here) the vacuum in the ampoules that were clamped-off was well maintained. As compared with the flame-sealed ampoules, sometimes a decrease in viability was found in the clamped-off ampoules, probably due to traces of air which diffused through the rubber connexions. However, this decrease in the controls was usually so small that it could be neglected in comparison with the mortality observed in the test ampoules when filled with air.

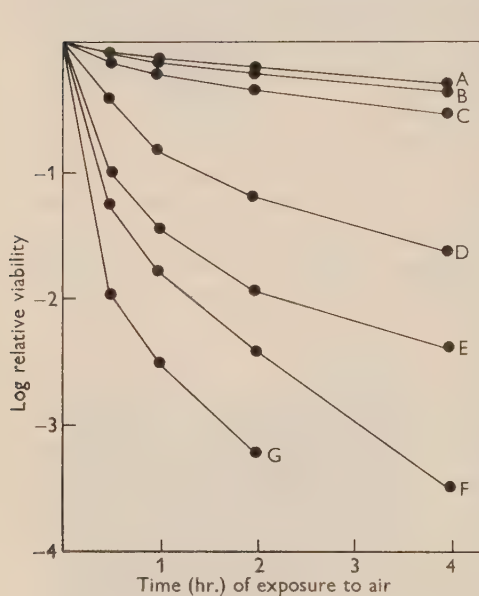


Fig. 1

Fig. 1. Viability of *Escherichia coli*, freeze-dried in single components of Naylor's medium, after exposure to dry air at 28°. A, complete medium; B, 1% (w/v) thiourea; C, 0.5% (w/v) thiourea; D, 0.5% (w/v) ammonium chloride; E, 0.5% (w/v) ascorbic acid neutralised to pH 6.5 with NaOH; F, distilled water; G, 2% (w/v) dextrin.

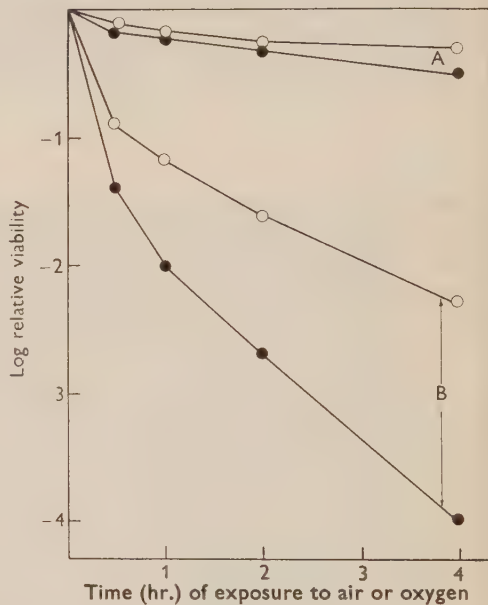


Fig. 2

Fig. 2. Viability of *Escherichia coli* strain B/r, freeze-dried from distilled water or 1% (w/v) thiourea, after exposure to dry air or oxygen at 28°. A, bacteria dried in 1% (w/v) thiourea; B, bacteria dried in water; O, exposed to air; ●, exposed to oxygen.

RESULTS

The Naylor & Smith (1946) medium was first examined for the ability of its components to protect dried *Escherichia coli* against the oxygen of air. The Naylor & Smith medium is composed of (% w/v): sodium ascorbate, 0.5; thiourea, 0.5; ammonium chloride, 0.5; dextrin, 2; in aqueous solution. Although ascorbic acid was originally contained in the Naylor & Smith medium as an antioxidant, its protective effect against air was negligible. Drying in dextrin gave worse results than drying from water, and only thiourea showed a protective power comparable to that of the complete medium. Increasing the concentration of thiourea from 0.5% (w/v, as in the Naylor & Smith medium) to 1% (w/v) permitted the replacement of the com-

plete medium by thiourea alone as far as the oxygen effect was concerned. Beside thiourea only ammonium chloride showed some protective action, although it was far less effective than thiourea. Fig. 2 shows the protection by 1% (w/v) thiourea for dried *Escherichia coli*, strain B/r, against air and oxygen. Table 1 shows the protection afforded by various derivatives and analogues of thiourea. As it could be assumed that thiourea acted in its tautomeric form either as a reducing agent or as a sulphhydryl compound or both, a few materials of these two classes were tested (Table 1, B). While ascorbic acid had hardly any protective action and cysteamine none at all, cysteine and especially sodium dithionite and reduced glutathione enhanced the lethal effects of oxygen considerably. The killing effect of these substances was pronounced in the presence of air only; dried bacteria *in vacuo* were not affected by them, either during lyophilization or after termination of the freeze-drying.

Ethylenediaminetetra-acetate (0.1%, w/v, pH 7), tested as a representative chelating agent, did not show any protective action. Some materials containing high-molecular compounds, namely, nutrient broth (double strength), skimmed milk and gelatin were also examined. They had no protective effect.

Glucose has proved to be an effective protecting agent in lyophilization (Fry & Greaves, 1951). Considering that at least part of the killing of bacteria during freeze-drying is due to oxygen, one would expect glucose to protect against this gas. Indeed, glucose showed protective properties. Other sugars and sugar derivatives were also tested (Table 1). As it seemed possible that a correlation might exist between the protective effect of a sugar by the given bacterial strain and its fermentability, fermentation tests were carried out according to standard bacteriological procedures (Mackie & McCartney, 1945). Glucose, galactose, mannose, fructose and lactose were fermented; sucrose, maltose and α -methylglucoside were not. The protective power of α -methylglucoside (not fermented) was as high as that of glucose (fermented); but since no trace of fermentation of α -methylglucoside was observed even after incubation for 3 days, its protective action appears not to be related to the free 1-position of the monosaccharide.

As already mentioned, ammonium chloride had some protective effect. Naylor & Smith (1946) mentioned that ammonium chloride could to a certain extent be replaced by sodium chloride in their freeze-drying medium. We tested various inorganic salts for protective effects against oxygen. The results are given in Table 1, E. The iodides of sodium and potassium proved to be the most effective protective agents found so far; hardly any killing occurred in the dried organisms when exposed to air in the presence of iodide. Sodium and potassium nitrites and thiocyanates were also very effective. Not always, however, was the cation without influence. Sodium chloride, bromide and nitrate were much more effective protective agents than the corresponding potassium salts. Lithium, and especially magnesium chloride, showed strong protective action, while the chlorides of calcium, ammonium and rubidium were much less effective. The protective action of the magnesium and sodium ions disappeared with the sulphate and phosphates. Both potassium and sodium fluoride appeared to enhance the oxygen effect.

Table 1. *Viability of Escherichia coli exposed to dry air at 28° for various periods in the presence of different media*

Drying medium	Relative viability of bacteria (%)					
	At the end of drying*	Kept after drying at 28° in dry air†				4 hr. in vacuum
		0.5 hr.	1 hr.	2 hr.	4 hr.	
Group A agents (all 0.13 M)						
Thiourea	83	77	64	47	37	100
Monomethylthiourea	94	88	76	73	59	100
Symmetrical dimethylthiourea	81	67	52	49	39	91
Asymmetrical dimethylthiourea	62	19	11	9	4	62
Trimethylthiourea	64	36	22	8	6	78
Ethylisothiuronium bromide HBr	88	40	23	14	4	81
Urea	79	40	33	11	8	66
Urethane	45	16	12	4	1	69
Thioacetamide	75	49	38	16	5	91
Acetamide	74	20	12	3	1	26
Thiosemicarbazide	59	13	6	3	1	54
Group B agents						
Na ascorbate (pH 6.5, 0.5%, w/v)	61	9	3	1	0.5	67
Na dithionite (0.05 M)	49	0.2	< 10 ⁻³	< 10 ⁻³	< 10 ⁻³	42
Cysteamine (pH 6.5, 0.13 M)	77	13	3	0.4	0.01	78
Cysteine (pH 6.5, 0.06 M)	64	3	0.3	0.02	< 10 ⁻³	87
Glutathione (0.16 M)	61	—	—	< 10 ⁻⁴	< 10 ⁻⁵	—
Glutathione (0.08 M)	60	—	—	< 10 ⁻⁴	< 10 ⁻⁵	—
Group C agents (all 0.11 M)						
D-Glucose	58	95	74	53	41	91
D-Galactose	70	57	47	38	31	97
D-Mannose	72	72	65	57	42	87
D-Fructose	60	40	35	36	25	93
L-Arabinose	91	58	51	38	31	75
Lactose	67	9	2	0.2	0.04	—
Sucrose	73	15	3	0.9	0.5	67
Maltose	66	35	1	0.3	0.2	50
Cellobiose	61	21	10	9	1	59
Melibiose	61	14	7	3	2	54
Group D agents (all 0.11 M)						
α-Methylglucoside	69	65	54	45	32	100
α-Methylmannoside	88	59	47	39	30	100
Mannitol	73	50	37	18	13	84
Inositol	76	33	23	11	3	62
Glucosamine HCl	87	71	65	51	42	98
Group E agents (0.16 M with exceptions)						
NaCl	63	81	62	40	30	75
KCl	76	7	2	0.8	0.2	80
NaBr	86	50	36	22	10	81
KBr	84	14	6	2	0.4	58
NaI	95	95	91	84	80	87
KI	92	93	86	79	82	96
NaF	37	< 10 ⁻²	< 10 ⁻³	< 10 ⁻³	< 10 ⁻⁴	10
KF	65	< 10 ⁻²	< 10 ⁻³	< 10 ⁻³	< 10 ⁻⁴	10
NaNO ₃	89	82	69	63	54	90
KNO ₃	82	17	9	4	0.7	48
NaNO ₂	75	84	79	64	44	75
KNO ₂	89	71	67	71	68	72
NaSCN	93	71	67	51	45	93
KSCN	80	76	65	62	52	96
Na ₂ SO ₄	48	2	0.2	—	—	30
K ₂ SO ₄	33	1	0.1	—	—	37
NaH ₂ PO ₄ (M/15) + Na ₂ HPO ₄	43	3	0.4	0.04	0.01	—
MgCl ₂ (0.10 M)	66	73	63	43	36	67
MgSO ₄ (0.10 M)	41	16	5	2	0.5	18
LiCl	77	46	31	21	12	74
RbCl	75	21	10	5	1	63
NH ₄ Cl	63	28	18	7	2	41
CaCl ₂ (0.10 M)	53	21	13	5	3	—

* % viability referred to viability before drying.

† % viability referred to viability at the end of drying, before exposure to air.

DISCUSSION

The substances tested in this study which protected freeze-dried bacteria from atmospheric oxygen may be conveniently divided into three groups. The first group comprises thiourea and its derivatives and analogues. Comparing the effectiveness of the different methyl derivatives, one sees that the exchange of more than one hydrogen in one or both amino groups of thiourea caused a substantial decrease of protecting power. This was especially evident with the two isomeric dimethyl derivatives; the symmetrical compound was much more effective than the asymmetrical one. Derivatives of isothiurea seemed to possess much less protective ability than thiourea itself. The replacement of sulphur by oxygen, as in passing from thiourea to urea or from thioacetamide to acetamide, decreased the protective power. When one of the amino groups was replaced by methyl (as in thioacetamide) or the hydrazine radical (as in thiosemicarbazide) the protective effect was practically nil. It is difficult to draw definite conclusions from these data about the mode of action of this first group of agents. Perhaps it may be thought that the protective effect is based on the binding of the agent at the site which is sensitive to oxygen, e.g. through a chelation mechanism. It is obvious, in any event, that the reducing property of thiourea and the other compounds found to be effective is not involved in the mode of action, since other similar reducing sulphydryl compounds were either inactive (cysteamine) or even enhanced the deleterious effect of oxygen (dithionite, cysteine, glutathione).

The second group of protective agents comprised the sugars and sugar derivatives. The disaccharides were much less effective than the monosaccharides; at the concentration tested some of the disaccharides were quite ineffective. No correlation was found between the protective efficiency of a sugar and its chemical and steric structure, its fermentability by the bacteria and its reducing power. α -Methylglucoside was not fermented, is non-reducing and was an effective protective agent, while sucrose was not fermented, is non-reducing, and did not protect. Lactose is reducing, was fermented but inactive; glucose and galactose are reducing, were both fermented and possessed good protective power.

The inorganic salts constituted the third group of protective compounds. In some cases, the sodium salt was much more effective than the potassium salt; here the protective action might be ascribed to the cation. On comparing the different chlorides, the smaller cations (lithium, sodium, magnesium) were more potent than potassium, calcium, ammonium or rubidium. Nevertheless, the action of the cation was affected by the anion to which it was linked. This is evident when the protective sodium chloride, bromide and nitrate are compared with the inactive sodium sulphate and phosphate; the same trend was apparent as between magnesium sulphate and the chloride. With the iodides, which are the best protective agents found so far, the sodium and potassium iodides protected equally well. This was also true of the nitrites and thiocyanates tested. In these cases the anion seems to be responsible for the protective action. We are at present unable to suggest a theory that would interpret correctly the effects of all the protective agents found so far. One has to consider the possibility that the different compounds affect the outcome of the reaction between oxygen and dried bacteria at different stages of the process, so that their modes of protective action might be different.

This paper forms part of a thesis submitted by one of us (M. B. L.) to the Hebrew University in partial fulfilment of the requirements for the degree of Ph.D.

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The Oxidase Reaction as a Taxonomic Tool

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SUMMARY

The oxidase test of Kovacs (1956) was applied to 1660 strains of various genera, of which 210 gave a positive reaction and 64 gave a delayed positive reaction. Members of the Neisseriaceae and Pseudomonadaceae were generally oxidase-positive, whereas members of the Enterobacteriaceae and, with few exceptions, Gram-positive organisms, were oxidase-negative; equivocal results were obtained in the Brucellaceae and the genus *Bacillus*. Kovacs test is simple and quickly performed, but very sensitive. Some taxonomic implications of the results are discussed.

INTRODUCTION

The oxidase reaction, based upon the ability of certain bacteria to produce indophenol by the oxidation of dimethyl-*p*-phenylenediamine and α -naphthol, was introduced by Gordon & McLeod (1928) to aid identification of gonococci, although specificity for this organism was not claimed. A 1-1.5 % (w/v) solution of dimethyl-*p*-phenylenediamine hydrochloride was poured over suspected colonies grown on a suitable medium in a Petri plate; oxidase-positive colonies developed a pink colour which successively became maroon, dark red and black in 10-30 min. The use of the more sensitive and less toxic tetramethyl compound was advocated by Ellingworth, McLeod & Gordon (1929). Kovacs (1956) smeared bacterial growth on filter-paper impregnated with 1 % (w/v) aqueous tetramethyl-*p*-phenylenediamine dihydrochloride solution. Oxidase-positive organisms produced a purple coloration on the paper within 10 sec. Kovacs reported that, apart from *Pseudomonas aeruginosa*, the common Gram-negative rods did not produce this coloration. Gaby & Hadley (1957) introduced their 'cytochrome oxidase' test in which a broth culture was used. The appearance of a blue colour on addition of aqueous dimethyl-*p*-phenylenediamine oxalate and ethanolic α -naphthol solutions indicated the presence of this enzyme. This test was modified by Ewing & Johnson (1960) to permit the use of agar slope cultures. Kovacs (1956) oxidase test has now been applied to some of the organisms held in the National Collection of Type Cultures and the results are reported here.

METHODS

Organisms. A total of 1660 strains has been tested. The organisms were grown on the surface of solid media at their optimum temperature for 18-24 hr. or until distinct colonies were obtained.

Oxidase test. Growth was picked off the medium with a platinum loop and rubbed on filter-paper impregnated with 1 % (w/v) aqueous tetramethyl-*p*-phenylene-

diamine dihydrochloride solution. Production of a purple colour within 10 sec. was recorded as positive, its development in 10–60 sec. as delayed positive, and the absence of coloration or its still later development as negative.

Sensitivity of test. Traces of iron catalyse the oxidation of the phenylenediamine compound with the production of a purple colour, which may give rise to false reactions, so that it is essential to use a platinum loop or wire to remove the bacterial growth for testing. The reagent loses its sensitivity and becomes discoloured on storage. A fresh batch was prepared every two weeks and kept in a glass-stoppered bottle, protected from light, in a refrigerator.

RESULTS

Of the 1660 strains tested 210 gave positive and 64 gave delayed reactions (Table 1).

Table 2 gives details of genera in which prompt positive results were obtained. Delayed positive reactions were common in the genera *Bacillus*, *Brucella*, *Haemophilus*, and *Pasteurella*.

Table 1. *Distribution of oxidase activity among bacterial genera*

Total number of strains tested was 1660; of these 210 were positive, 64 were delayed positive and 1386 were negative.

Genus	No. of strains tested	No. of strains			Genus	No. of strains tested	No. of strains		
		+	(+)	—			+	(+)	—
<i>Achromobacter</i>	18	2	4	12	<i>Neisseria</i>	57	57	.	.
<i>Actinobacillus</i>	7	.	3	4	<i>Nocardia</i>	9	.	.	9
<i>Actinomyces</i>	13	.	.	13	<i>Pasteurella</i>	62	15	10	37
<i>Aerococcus</i>	6	.	.	6	<i>Proteus</i>	40	.	.	40
<i>Aeromonas</i>	5	5	.	.	<i>Pseudomonas</i>	33	32	1	.
<i>Alcaligenes</i>	7	7	.	.	<i>Salmonella</i>	97	.	.	97
<i>Arizona</i>	80	.	.	80	<i>Serratia</i>	12	.	.	12
<i>Bacillus</i>	84	2	21	61	<i>Shigella</i>	89	.	.	89
<i>Bordetella</i>	23	19	1	3	<i>Staphylococcus</i>	97	.	1	96
<i>Brucella</i>	31	14	6	11	<i>Streptococcus</i>	130	.	.	130
<i>Chromobacterium</i>	6	.	.	6*	<i>Streptomyces</i>	6	.	.	6
<i>Citrobacter</i>	17	.	.	17	<i>Vibrio</i>	35	32	.	3
<i>Clostridium</i>	54	.	.	54					
<i>Corynebacterium</i>	91	.	.	91	Miscellaneous bacteria				
<i>Enterobacter</i> †	17	.	.	17	<i>Bacteroides necrophorus</i>	1	.	.	1
<i>Erysipelothrix</i>	7	.	.	7	<i>Coccobacillus mycetoides</i>	1	.	.	1
<i>Escherichia</i>	107	.	.	107	<i>Flavobacterium meningosepticum</i>	1	1	.	.
<i>Haemophilus</i>	33	5	13	15	<i>Jensenia canicruria</i>	1	.	.	1
<i>Hafnia</i>	11	.	.	11	<i>Kurthia zopfii</i>	2	.	.	2
<i>Klebsiella</i>	274	.	.	274	<i>Leptotrichium dentium</i>	1	.	.	1
<i>Lactobacillus</i>	7	.	.	7	<i>Noguchia granulosis</i>	1	.	.	1
<i>Leuconostoc</i>	3	.	.	3	<i>Pediococcus cerevisiae</i>	1	.	.	1
<i>Listeria</i>	9	.	.	9	<i>Polysepta pedis</i>	1	.	.	1
<i>Loefflerella</i>	15	8	3	4	' <i>Pseudomonas iodinum</i> '	1	.	1	.
<i>Lophomonas</i>	4	4	.	.	<i>Sarcina</i> sp.	1	.	.	1
<i>Moraxella</i>	10	7	.	3	<i>Spirillum rubrum</i>	1	.	.	1
<i>Mycobacterium</i>	40	.	.	40	<i>Streptobacillus moniliformis</i>	1	.	.	1

* See discussion.

+ = positive reaction within 10 sec.; (+) = delayed positive reaction in 10–60 sec.; — = negative reaction.

† The generic name *Enterobacter* was proposed by Hormaeche & Edwards (1960) to replace the generic name *Cloaca*. The genus as now proposed contains two species, *E. cloacae* and *E. aerogenes*.

Oxidase, catalase and aerobiosis

From the results in Tables 1 and 2 it is seen that the oxidase-positive strains were all aerobes or facultative anaerobes, with the exception of *Vibrio fetus* which is micro-aerophilic. Gordon & McLeod (1928) believed the oxidase reaction to be restricted to organisms which grew best in the presence of oxygen and which formed catalase. In a study of organisms resembling *Alcaligenes faecalis*, Moore & Pickett (1960) found 9 oxidase-positive strains which were catalase-negative, but of the organisms studied here all oxidase-positive strains produced catalase. None of the strict anaerobes showed any oxidase activity. Its absence from these organisms and from the Lactobacillaceae is in agreement with the lack of cytochrome in these organisms (Deibel & Evans, 1960).

Table 2. *Species within the oxidase-positive genera of bacteria*

Species	No. of strains	+	(+)	-	Species	No. of strains	+	(+)	-
<i>Achromobacter anitratus</i>	11	.	.	11	<i>Moraxella liquefaciens</i>	1	1	.	.
<i>A. equuli</i>	7	2	4	1	<i>M. twoffii</i>	3	.	.	3
<i>Aeromonas hydrophila</i>	3	3	.	.	<i>Neisseria animalis</i>	1	1	.	.
<i>A. liquefaciens</i>	1	1	.	.	<i>N. catarrhalis</i>	7	7	.	.
<i>A. salmonicida</i>	1	1	.	.	<i>N. flavescens</i>	3	3	.	.
<i>Alcaligenes denitrificans</i>	1	1	.	.	<i>N. gonorrhoeae</i>	12	12	.	.
<i>A. faecalis</i>	5	5	.	.	<i>N. meningitidis</i>	32	32	.	.
<i>A. viscosus</i>	1	1	.	.	<i>N. pharyngis</i>	2	2	.	.
<i>Bordetella bronchiseptica</i>	12	12	.	.	<i>Pasteurella haemolytica</i>	6	6	.	.
<i>B. parapertussis</i>	3	.	.	3	<i>P. pestis</i>	10	.	.	10
<i>B. pertussis</i>	8	7	1	.	<i>P. pseudotuberculosis</i>	20	.	.	20
<i>Brucella abortus</i>	12	8	3	1	<i>P. septica</i>	26	9	10	7
<i>B. melitensis</i>	6	3	2	1	<i>Pseudomonas aeruginosa</i>	14	14	.	.
<i>B. neotomae</i>	4	.	.	4	<i>P. chlororaphis</i>	1	1	.	.
<i>B. ovis</i>	1	.	.	1	<i>P. diminuta</i>	1	1	.	.
<i>B. paramelitensis</i>	1	1	.	.	<i>P. fluorescens</i>	5	5	.	.
<i>B. suis</i>	7	2	1	4	<i>P. graveolens</i>	1	1	.	.
<i>Haemophilus aegyptius</i>	3	.	2	1	<i>P. mucidolens</i>	1	1	.	.
<i>H. aphrophilus</i>	4	.	.	4	<i>P. ovalis</i>	2	1	1	.
<i>H. canis</i>	2	.	1	1	<i>P. polycolor</i>	1	1	.	.
<i>H. gallinarum</i>	1	.	.	1	<i>P. synchyanea</i>	1	1	.	.
<i>H. haemolyticus</i>	1	.	1	.	<i>Pseudomonas</i> spp.	6	6	.	.
<i>H. influenzae</i>	16	4	7	5	<i>Vibrio cholerae asiaticae</i>	15	15	.	.
<i>H. parainfluenzae</i>	2	.	.	2	<i>V. el Tor</i>	10	10	.	.
<i>H. suis</i>	4	1	2	1	<i>V. fetus</i>	1	1	.	.
<i>Loefflerella mallei</i>	5	.	1	4	<i>V. metchnikovii</i>	1	.	.	1
<i>L. pseudomallei</i>	10	8	2	.	<i>V. paracholerae</i>	1	1	.	.
<i>Lophomonas alcaligenes</i>	4	4	.	.	<i>V. percolans</i>	1	1	.	.
<i>Moraxella bovis</i>	3	3	.	.	<i>V. proteus</i>	2	.	.	2
<i>M. lacunata</i>	3	3	.	.	<i>Vibrio</i> spp.	4	4	.	.

+ = positive reaction; (+) = delayed positive reaction; - = negative reaction.

Gram-positive organisms

With the exception of 'Pseudomonas iodinum', one strain of *Staphylococcus lactis*, and some *Bacillus* spp., oxidase activity was not found among Gram-positive organisms.

Oxidase activity in *Bacillus anthracis* and *B. subtilis* was recorded by Gordon & McLeod (1928) and Price (1929). With Kovacs technique, few prompt positives but

several delayed reactions were obtained among strains of the 22 species of the genus *Bacillus* tested here.

One strain of *Bacillus laterosporus* gave a positive reaction, one a delayed positive and one a negative reaction; with 7 strains of *B. licheniformis*, 1 positive, 2 delayed positive and 4 negative reactions were obtained. Delayed positive reactions were obtained with 5 strains of *B. sphaericus*, 2 of *B. alvei* and one each of *B. freundenreichii* and *B. lentus*. Some strains of the following species gave delayed positive reactions: *B. anthracis* (2 of 4 strains tested), *B. brevis* (1 of 3), *B. cereus* (1 of 7), *B. mycoides* (1 of 3), *B. subtilis* (4 of 14).

Oxidase activity was not observed in the following species: *Bacillus alcalophilus* (1 strain), *B. carotarium* (1), *B. circulans* (4), *B. coagulans* (3), *B. loehnisii* (1), *B. macerans* (4), *B. megaterium* (6), *B. pantothenicus* (4), *B. polymyxa* (2), *B. pumilis* (7) and *B. stearothermophilus* (1).

Gram-negative organisms

Oxidase activity was more common among the Gram-negative organisms. All strains tested of the genus *Pseudomonas* were oxidase-positive, as found by Buttiaux & Gagnon (1958); however, in one strain of *P. ovalis* the reaction was delayed. The observation of Gould & McLeod (1960) that young unpigmented growth of *P. aeruginosa* gave a more intense oxidase reaction than older colonies was confirmed, Köhler (1959) reported *P. fluorescens* to be oxidase-negative but, like Klinge (1960), I could not confirm this.

Only 5 strains of *Aeromonas* were tested, but all were oxidase-positive, a result confirming Ewing & Johnson (1960) who tested 36 strains. All strains of *Achromobacter anitratus* were oxidase-negative but 7 strains of *A. equuli* (*Actinobacillus equuli*) gave equivocal results. Buttiaux & Gagnon (1958) found fewer oxidase-positive strains of *Aeromonas* (5 of 24), but 12 of 23 strains of *Achromobacter* and none of 7 strains of '*B. anitratum*' were positive.

Strains of the genus *Alcaligenes* all gave positive reactions, although the colour produced on the reagent-impregnated paper intensified greatly after contact for 10 sec. Ewing & Johnson (1960) found the genus to be oxidase-positive but they too examined only a few strains. Gaby & Free (1958) considered Kovacs test to be over-sensitive because strains of *A. faecalis* gave reactions similar to those of *Pseudomonas aeruginosa*.

With the exception of *Vibrio metchnikovii* and *V. proteus*, all strains tested of the genus *Vibrio* were oxidase-positive. Oxidase activity was found by Ewing & Johnson (1960) in all of 115 strains of *V. cholerae asiaticae* and in 12 other strains of the genus. The four strains of *Lophomonas alcaligenes* were oxidase-positive, a character of the species (Galarneault & Leifson, 1956). In the genus *Neisseria*, all strains tested gave a positive reaction.

Because of their violet pigment, which was absorbed into the filter paper, difficulty was encountered in determining the oxidase activity of *Chromobacterium* species. Tests made with non-pigmented colonies and young cultures before the pigment was well developed failed to show definite oxidase activity. In Table 1 these strains have therefore been recorded as oxidase-negative, although it may be possible to observe oxidase activity by other test methods. The one strain of *Flavobacterium meningosepticum* gave a positive oxidase reaction, as originally

reported by King (1959). Reports of oxidase activity in this genus and in *Xanthomonas* are conflicting (Buttiaux & Gagnon, 1958; Ewing & Johnson, 1960), mainly because of the small number of strains tested.

Of the total of 1660 strains tested in the present work, 744 were members of the Enterobacteriaceae and none of these gave a positive or even a delayed positive reaction. Oxidase activity was not demonstrated in 800 strains of this family by Buttiaux & Gagnon (1958) nor in 1222 strains tested by Ewing & Johnson (1960). Gordon & McLeod (1928) reported *Serratia marcescens* to be oxidase-positive, but no confirmation of this has been obtained.

The Brucellaceae ('Parvobacteriaceae') was the family most heterogeneous in oxidase activity. Two strains of *Actinobacillus actinomycetemcomitans* and one strain each of *Bacteroides necrophorus*, *Noguchia granulosis* and *Streptobacillus moniliformis* were oxidase-negative. Of 5 strains of *A. lignieresii*, 3 gave a delayed positive reaction when colonies from blood agar plates were tested, but very weak or negative results when serum glucose agar was used.

All strains of *Bordetella bronchiseptica* and *B. pertussis* were oxidase-positive (although in one strain of the latter the reaction was delayed 15 sec.) whereas those of *B. parapertussis* were negative. Gordon & McLeod (1928) found that stock strains of *B. pertussis* on Dorset egg medium gave a positive reaction but that fresh isolates were negative. They noted that uninoculated Bordet-Gengou medium gave a positive reaction on addition of the test reagent. Using the tetramethyl compound (Ellingworth *et al.* 1929), *B. pertussis* was found to be oxidase-positive. Lacey (1960) reported that both *B. bronchiseptica* and *B. pertussis* gave positive oxidase reactions within 30 sec. when a loopful of the reagent was applied to the bacterial growth. Lautrop (1960) considered these two species to be oxidase-positive within 15 sec. by Kovacs method; he also noted the possibility of false positives due to the high proportion of blood in many media for *B. pertussis*. For the tests reported here, this organism was grown on Bordet-Gengou medium, except for two phase IV strains which were grown on Lemco agar.

The older species of *Brucella* gave equivocal results whereas strains of two proposed new species, *B. neotomae* and *B. ovis*, did not show oxidase activity. It was not possible to distinguish American and Danish strains of *B. suis* on their oxidase activity. *B. melitensis* was reported by Gordon & McLeod (1928) to be oxidase-positive. In a quantitative study Richardson (1957) showed that species of this genus differed markedly in their cytochrome oxidase activity; typical strains of *B. abortus* had twice the activity of *B. melitensis* and more than twice that of *B. suis* or thionin-resistant strains of *B. abortus*. Strong peroxidase activity was reported (Kréméry, 1959) in *B. suis*, and Smith (1954) noted that *p*-phenylenediamines might be oxidized by peroxidases. It is thus possible that an 'oxidase' activity in *B. suis* may be a reflexion of this side reaction rather than of oxidase proper.

Equivocal results were also obtained in the genus *Haemophilus*; over half the strains tested showed some oxidase activity. With dimethyl-*p*-phenylenediamine, *H. influenzae* was originally reported to be oxidase-negative, but use of the more sensitive tetramethyl compound showed it to be positive (Ellingworth *et al.* 1929). With the exception of *H. influenzae*, few strains of the other species of this genus were tested in the present work. The 4 strains of *H. aphrophilus*, which were grown in an atmosphere of CO₂, were all oxidase-negative.

Strains of *Loefflerella pseudomallei* were all oxidase-positive, although in two strains the reaction was delayed (15 sec.), whereas 4 strains of *L. mallei* gave a negative and 1 strain a delayed (30 sec.) positive reaction. Gordon & McLeod (1928) reported *L. mallei* as oxidase-positive and Fournier (1959) reported *L. pseudomallei* to be slowly positive by Gaby & Hadley's method. Miller *et al.* (1948) reported both species to be oxidase-positive. With the exception of *Moraxella lwoffii*, all members of the genus *Moraxella* were oxidase-positive, this is in agreement with the results of Henriksen (1952).

Strains of *Pasteurella pseudotuberculosis* and *P. pestis* were devoid of oxidase activity. *P. pestis* has been reported as weakly oxidase-positive (Gordon & McLeod, 1928). Strains of *P. haemolytica* gave positive reactions whereas variable results were obtained with *P. septica*.

DISCUSSION

The oxidase reaction was originally thought to depend on the presence of both peroxide and peroxidase (Gordon & McLeod, 1928). It is now believed to be due to the presence of a cytochrome oxidase, the enzyme which catalyses the oxidation of reduced cytochrome by molecular oxygen and which acts as the terminal stage in electron transfer. Most aerobic and facultatively anaerobic bacteria contain a cytochrome system which acts as an electron carrier in aerobic respiration. Many of these organisms also produce catalase. The cytochromes are a group of respiratory pigments comprising a number of related iron-porphyrin compounds. The distribution of these cytochromes varies with different bacteria; some have several while others have only one or none at all. Most workers have used the visual spectro-scope to examine the absorption spectra of bacterial cytochromes, a technique which is not sufficiently sensitive to distinguish between cytochromes whose absorption maxima are close together. Smith (1954), in a review of bacterial cytochromes, pointed out the disagreement among various workers about the distribution of the cytochromes. Castor & Chance (1959) demonstrated the existence of four different cytochrome oxidases in various bacteria.

Many of the oxidase-positive organisms (Tables 1 and 2) have been reported in the past to contain cytochrome *c*, a component which is not autoxidizable but only oxidized in the presence of cytochrome *c* oxidase. The distribution, identity and nomenclature of the various cytochrome components remain confused, however. Consequently in this paper the term 'oxidase' is used without prejudice and no assumptions have been made about the possible identity of the material responsible for positive oxidase reactions with cytochrome *c* oxidase or any other component of the bacterial cell.

For a test to be of taxonomic value it is imperative that the results be reproducible with a standard technique. The oxidase test used at present differs in different laboratories both in the method and the reagents used. Gaby & Free (1958) claimed that the cytochrome oxidase test (Gaby & Hadley, 1957) was more accurate than Kovacs method, which they considered to be over-sensitive. Buttiaux & Gagnon (1958) found Gaby & Hadley's reaction to be suitable with *Pseudomonas* strains but not with other organisms which produced a slow coloration, making it difficult to decide whether it was a slow oxidase reaction or autoxidation of the reagent. Kovacs method, on the other hand, was rapid, simple and sensitive. Köhler (1959)

found a margin of error of up to 12 % in Gaby & Hadley's test and the observation time had to be increased to 20 min. Klinge is reported to have stated (Billing, 1960) that the number of strains of *P. aeruginosa* giving a positive reaction depended on the medium on which they were grown. Kovacs method, besides being simpler and quicker than the other methods, has the advantage that only a portion of a colony is needed for the test. It is undoubtedly a more sensitive method of determining oxidase activity than the other tests. The reagents used in the various methods include *p*-phenylenediamine hydrochloride, dimethyl-*p*-phenylenediamine (also known as *p*-aminodimethylaniline) hydrochloride or oxalate (Carpenter, Suhrlund & Morrison, 1947) and tetramethyl-*p*-phenylenediamine dihydrochloride, all with or without the addition of aniline or α -naphthol. The instability of solutions of the *p*-phenylenediamines is a disadvantage common to all test methods.

Taxonomic implications

Although in many genera only a few strains have been examined, the taxonomic implications of the results need appraisal. The genus *Aeromonas* has many of the characters of the Enterobacteriaceae; it differs in being oxidase-positive and polarly flagellate. It has been suggested (Stevenson, 1959) that all members of the genus *Aeromonas* are unpigmented members of the genus *Serratia*. Oxidase activity has not been found in strains of *Serratia* and there is no evidence that the pigment prodigiosin plays any role in respiration in a manner analogous to that of 'oxidase'; Stevenson's hypothesis is therefore not supported.

The genus *Alcaligenes* is not yet satisfactorily delineated and the literature abounds with contradictory statements about its oxidase activity. Türck (1952) found that her strains formed two groups, oxidase-positive vibrio-like forms and oxidase-negative non-motile rods. Buttiaux & Gagnon (1958) considered that *Alcaligenes* should be included in *Achromobacter*, as they could not find *Alcaligenes faecalis* to be motile. Klinge (1958) thought that 'Bacterium anitratum', B 5 W organism, 'Diplococcus mucosus' and similar organisms should be classified as *Alcaligenes*. He rejected pleomorphism as a criterion for classification but stressed the importance of a negative oxidase reaction. Moore & Pickett (1960) questioned the validity of the genus *Alcaligenes* because they found few isolates of *A. faecalis* which resembled the original description in having peritrichous flagella. Of their 40 strains, 20 were oxidase-positive and 2 weakly positive and they consider they should be included in the genus *Achromobacter*. Haupt (1957) listed *Achromobacter equuli*, which gave equivocal results in my tests, as *Actinobacillus equuli*. Accepting this, it may be suggested that it would be expedient for oxidase-positive organisms now in the genera *Achromobacter* or *Alcaligenes* to be classified as *Alcaligenes* and oxidase-negative organisms as *Achromobacter*. A strain of *Alcaligenes faecalis* (NCTC 415) designated as 'type' by Winslow, Kligler & Rothberg (1919) is oxidase-positive as is the type strain of *A. denitrificans*.

The absence of oxidase activity in *Moraxella lwoffii* distinguishes it from the other recognized species of *Moraxella*. Henriksen (1952) discussed the taxonomy of this genus and considered that it might be closely related to *Neisseria*, in a relation analogous to that between lactobacilli and streptococci. In a later paper (1960) he stated that *M. lwoffii* did not belong to the genus *Moraxella*. Klinge (1958) would

include *M. lwoffii* in the genus *Alcaligenes*, but from my results it would seem better to place it in the genus *Achromobacter*.

Brindle & Cowan (1951) demonstrated that the type of flagellation of *Loefflerella pseudomallei* warranted its inclusion in the Pseudomonadaceae; they concluded, however, that it was closely related to *L. mallei* and the two species should form a genus which should not be combined with any then accepted genus of the Pseudomonadaceae. Wetmore & Gochenour (1956) agreed that *L. pseudomallei* could be included in this family. Fournier (1959), however, considered that there was no phylogenetic relation between this organism and *Pseudomonas aeruginosa*, and that *L. pseudomallei* and *L. mallei* were species of the same genus. *Bergey's Manual* (1957) listed these two organisms in different genera, as *Pseudomonas pseudomallei* and *Actinobacillus mallei*. The demonstration of oxidase activity in the former and its virtual absence from the latter is additional evidence for the inclusion of *L. pseudomallei* in the Pseudomonadaceae and the exclusion of *L. mallei* from this family.

'*Pseudomonas iodinum*' is a non-motile, non-sporing Gram-positive rod. Sneath (1960) did not consider that it should be included in the genus *Pseudomonas* as it has the morphology of a diphtheroid; he thought that it may belong to the genus *Corynebacterium* or *Brevibacterium*. Its oxidase activity excludes it from the first genus, however, if absence of oxidase activity is a character of the genus as a whole. (Only members of this genus which are of medical or veterinary interest were examined.) Reports have not been found in the literature about oxidase determinations in species of *Corynebacterium* from plant sources or in species of *Brevibacterium*. For the present it is felt that this organism should be retained in the genus *Pseudomonas* until an 'oxidase spectrum' has been determined.

In addition to the organism tested here, the oxidase activity of some other genera has been reported. Ewing & Johnson (1960) found only 3 slow and weak positives among 34 strains of *Erwinia* tested. Oxidase activity has been demonstrated in some strains of *Veillonella* (Berger, 1960), and in some leptospires (Goldberg & Armstrong, 1959; Faine, 1960) although Czekalowski, McLeod & Rodican (1953) obtained equivocal results with these organisms.

The results reported here confirm the claims made by various workers that the oxidase test is of value in distinguishing between different groups of organisms. Previously attention has been directed to the Enterobacteriaceae and the Pseudomonadaceae, but evidence is now accumulating that the test can be of value in other groups. Of the methods for carrying out the test that of Kovacs is reliable so long as freshly prepared reagent is used and a time limit for positives is set which is in accordance with the degree of sensitivity required.

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Inhibition of Enzyme Formation Following Infection of *Escherichia coli* with phage T2r⁺

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SUMMARY

The synthesis of aspartate carbamyl transferase (ureidosuccinic synthetase), of dihydroorotic dehydrogenase and of alkaline phosphatase by a uracil-requiring strain of *Escherichia coli*, when the organisms were suspended in a minimal medium lacking uracil, was followed subsequent to infection of the cells with bacteriophage T2r⁺ or with ghosts of the bacteriophage particles. The results were compared with the synthesis of these enzymes in uninfected cells. Following infection, the formation of all three enzymes was halted. Supplementation of the medium with casein hydrolysate and tryptophan or with 0.01 M-Mg⁺⁺ + 10⁻³ M-spermine did not prevent the inhibition caused by bacteriophage infection.

INTRODUCTION

When *Escherichia coli* in logarithmic growth is infected with phage T2, the net synthesis of protein continues but at a linear rather than a logarithmic rate (Cohen, 1948). The majority of the protein synthesized appears to be phage protein or enzymes concerned with phage formation (Bessman, 1959; Sommerville, Ebisuzaki & Greenberg, 1959; Kornberg, Zimmerman, Kornberg & Josse, 1959; Keck, Mahler & Fraser, 1960). Whether a cell infected by a lytic phage is capable of synthesizing protein not concerned directly with phage synthesis was examined earlier by Monod & Wollman (1947) and by Benzer (1953). They found that β -galactosidase was not formed by these cells following the addition of the inducer, lactose. French & Siminovitch (1955) also found that this enzyme was not formed following infection with phage ghosts. Sher & Mallette (1954) also were unable to demonstrate the continued formation of the inducible lysine decarboxylase after addition of either phage T2r⁺ or phage ghosts. However, even under conditions where limited amounts of protein can be synthesized, bacteria are able to synthesize relatively large amounts of enzymes in response to the removal of a repressor substance for these enzymes from the medium (Gorini & Maas, 1957; Vogel, 1957; Yates & Pardee, 1957; Levin & Magasanik, 1961). By using such a system, we have examined the effect of phage infection on the ability of *Escherichia coli* to synthesize the enzymes aspartate carbamyl transferase (ureidosuccinic synthetase), dihydroorotic dehydrogenase and alkaline phosphatase after removal of uracil and inorganic phosphate from the medium. These enzymes were chosen because under certain circumstances they appear to be essential for the formation of nucleic acid precursors.

METHODS

Organism. Strain B₃₉, a uracil-requiring mutant of *Escherichia coli* B, obtained from Dr F. C. Neidhardt (Harvard Medical School, Boston, Mass., U.S.A.), was used in these studies.

Bacteriophage. Coli phage strain T2r⁺ maintained in this laboratory was used as the infecting agent and as the source of phage ghosts. Phage T2r⁺ stocks were prepared from lysates of bacteria grown in the basal salts medium and were purified by the method of Hook *et al.* (1946).

The ghosts were prepared according to the procedure of Herriott & Barlow (1957). The protein content of ghost suspensions and of the starting phage preparation were measured; the % protein remaining in the ghost suspension multiplied by the phage titre was accepted as the ghost titre. The viable phage titre of the ghost suspension was less than 0.2 % of the original phage titre and was less than 0.5 % of the ghost titre.

Media. Bacterial cultures were maintained on agar slopes containing (% w/v): Tryptone, 1.0; yeast extract, 0.5; Na₂HPO₄·2H₂O, 0.3; KH₂PO₄, 0.1; glucose, 0.5; agar, 2.0. The cultures were transferred to fresh medium at 2-week intervals.

The composition of the basal medium used for growing and infecting the bacteria was (% w/v): NH₄Cl, 0.1; Na₂HPO₄·2H₂O, 1.05; KH₂PO₄, 0.45; MgSO₄·H₂O, 0.01; NaCl, 0.1. Glucose added to a final concentration of 0.2 % (w/v) was used as the carbon and energy source; uracil when added to the medium was at 50 µg./ml.

When a medium lacking inorganic phosphate was desired 2-amino-2-hydroxy-methylpropane-1:3-diol (tris) adjusted to pH 7.4 with HCl was used to buffer the medium. The final concentration of tris was 0.1 M.

The phage particles were stored in a diluting fluid composed of (% w/v): NaCl, 0.65; MgCl₂·6H₂O, 0.2; gelatin, 0.07.

Nutrient agar was used for performing phage assays according to the agar layer technique of Adams (1950).

Chemicals. Uracil and L-tryptophan were obtained from L. Light & Co., L-dihydro-otic acid and tris buffer were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.; ureidosuccinic acid and paranitrophenylphosphate were obtained from British Drug Houses, Ltd, spermine tetrahydrochloride was obtained from Hofmann La Roche, Basle, Switzerland. Tryptone, yeast extract and casein hydrolysate were obtained from Oxo, Ltd. Dilithium carbamylphosphate was generously provided by Mr T. Gascoyne of this Department. All other chemicals were of reagent grade.

Preparation of suspensions of organisms. *Escherichia coli* B, strain B₃₉, was grown overnight at 37° with forced aeration in the basal medium containing uracil. Fifteen ml. of this culture were used to inoculate 600 ml. fresh medium of the same composition, and the culture was then grown for three to four generations under similar conditions. The organisms, which were growing logarithmically, were centrifuged down, washed twice with basal salts medium and then resuspended in one-half to three-quarters of the initial volume of basal medium. The viable counts of these suspensions were from 8×10^8 to 2×10^9 organisms/ml. Portions (25 ml.) of these suspensions were distributed into 100 ml. Erlenmeyer flasks and after the flasks were placed in a water bath at 37°, the cultures were incubated with forced aeration for the desired length of time and with the necessary additions. For the studies on alkaline phosphatase for-

mation, organisms were washed and resuspended in the tris buffer medium; subsequent steps were as described above.

Preparation of enzyme extracts and determination of activities. Portions (20–25 ml.) of culture were centrifuged, the deposit washed with cold distilled water and then resuspended in 5.0 ml. cold water. The suspension was then disrupted for 4–5 min. in a 19 kc. 60 W. sonic oscillator (Mullard-Measuring and Scientific Equipment Ltd., London). This preparation was then centrifuged for 5 min. at 12,000*g* and at 0–5° in an International refrigerated centrifuge. The supernatant solution was decanted and tested for its aspartate carbamyl transferase and dihydroorotic dehydrogenase activities by the procedures of Yates & Pardee (1957). Extracts for the determination of alkaline phosphatase activity were prepared and tested according to the procedure of Torriani (1960). Protein was measured by the method of Lowry, Rosebrough, Faar & Randall (1951).

RESULTS

Aspartate carbamyl transferase

Yates & Pardee (1957) demonstrated rapid synthesis of aspartate carbamyl transferase by uracil-requiring auxotrophs following exhaustion of uracil from the medium in which the organisms had been growing. When *Escherichia coli* B, strain B₃₉, was incubated in basal medium lacking uracil, there was an increase with time in the enzymic activity of the cell extracts. This increase in activity depended on the presence of a source of nitrogen in the basal medium (Fig. 1, curve A). More rapid synthesis of enzyme occurred following the addition of amino acids in the form of casein hydrolysate + tryptophan than occurred after the addition of the simple nitrogen source NH₄Cl (Fig. 1, curves E and B).

Table 1. *Aspartate carbamyl transferase activity of extracts from uninfected and infected cells of Escherichia coli B, strain B₃₉*

62.5 μ g. of protein of an extract prepared from uninfected cells was used in A and C. 72.5 μ g. of protein of an extract from infected cells was used in B and C. The reaction mixtures were incubated for 60 min. at 25°. The activity in C was 92.5 % of the sum of the activities of A and B.

Extract	Ureidosuccinic acid formed (μ mole)
A. Uninfected cells	5.7
B. Infected cells	2.4
C. A+B	7.5

The effect of phage T2r⁺ on the synthesis of the enzyme is shown also in Fig. 1. Addition of phage T2r⁺ 5 min. after suspending the organisms in uracil-free medium prevented the continued synthesis of enzyme normally found in uninfected cultures. This complete inhibition could be demonstrated despite different rates of synthesis proceeding at the time of infection (Fig. 1, curves D and G). Attempts to increase the rate of enzyme synthesis at the time of infection also failed to prevent the complete inhibition (Fig. 1, curve C₁ compared with curve D). The addition of ghosts also caused a complete inhibition of enzyme synthesis (Fig. 1, curves C₂ and F).

The possibility that phage infection prevented the appearance of increased activity of extracts by causing the production of an inhibitor of enzyme action, rather than by

causing an inhibition of enzyme formation, proved unlikely since it was found that the addition of an extract from infected organisms to an extract from uninfected organisms changed only slightly the activity of the total preparation as compared with the sum of the two separate activities (Table 1).

That phage infection of *Escherichia coli* B, strain B₃₉, caused the formation of uracil, just as phage infection of *E. coli*, strain 15T⁻, caused the formation of thymine (Barner & Cohen, 1954), and that the uracil formed subsequently prevented the synthesis of the enzyme appeared unlikely since no change occurred in the total number of infective centres in a culture of *E. coli* B, strain B₃₉, infected in the absence of uracil and incubated for 95 min. (Fig. 2).

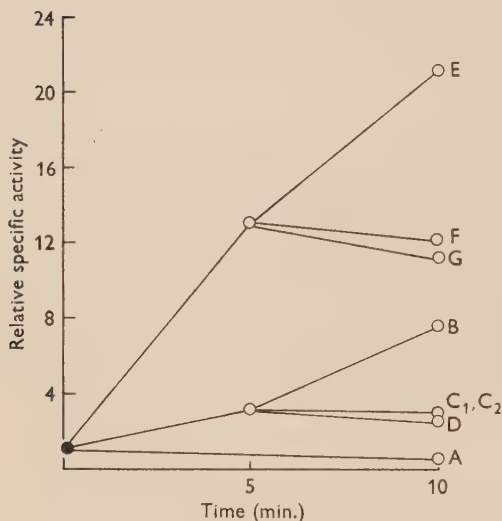


Fig. 1

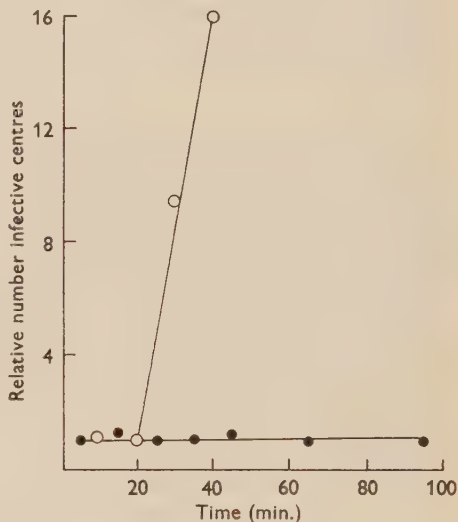


Fig. 2

Fig. 1. The effect of nitrogen sources, bacteriophage and ghosts on aspartate carbamyl transferase formation in *Escherichia coli* B, strain B₃₉. The values plotted are the specific activities of extracts relative to zero time. Specific activities are the μ mole ureidosuccinic acid formed/mg. protein/hr. Curves: A, no nitrogen source added; B, NH_4Cl (0.2 %) added at zero time; C₁, T2r⁺, casein hydrolysate (0.2 %, w/v) + tryptophan (0.1 %, w/v) added at 5 min.; C₂, ghosts added at 5 min.; D, phage T2r⁺ added at 5 min.; E, casein hydrolysate (0.2 %, w/v) + tryptophan (0.1 %, w/v) added at zero time; F, ghosts added at 5 min.; G, phage T2r⁺ added at 5 min. The multiplicities of infection were: C₁, 4.7; C₂, 6.0; D, 7.9; F, 2.4; G, 3.9.

Fig. 2. The total number of infective centres of phage T2r⁺ relative to the number added to a culture of *Escherichia coli* B, strain B₃₉. (●), No uracil in the medium; (○), uracil added. Bacterial count = 1.6×10^6 organisms/ml. Multiplicity of infection, 0.01. For other details of procedure see Burton (1955).

High multiplicities of infection can cause cell lysis (Delbrück, 1940) and thus release repressor molecules into the medium. Therefore, a medium was chosen in which one might expect the degree of cell lysis to be lower and consequently in which one might expect the apparent inhibition of enzyme formation caused by bacteriophage infection to be prevented. Infection was carried out in a medium containing 0.01 M- Mg^{++} and in a medium containing 0.01 M- Mg^{++} + 10^{-3} M-spermine. The addition of spermine to a basal medium has been shown to maintain spheroplasts of

Escherichia coli (Mager, 1959). In neither case was synthesis of enzyme carried out (Fig. 3). The inhibition of enzyme formation in the medium containing 0.01 M-Mg⁺⁺ remained complete for as long as 90 min. (curve E) despite the fact that no viable phage particles were formed during this period.

Dihydroorotic dehydrogenase

Dihydroorotic dehydrogenase also has been found to increase in pyrimidine auxotrophs maintained in the absence of uracil (Yates & Pardee, 1957). The formation of this enzyme by infected and uninfected organisms of *Escherichia coli* B, strain B₃₉, was examined; results similar to those found with aspartate carbamyl transferase were obtained (Table 2).

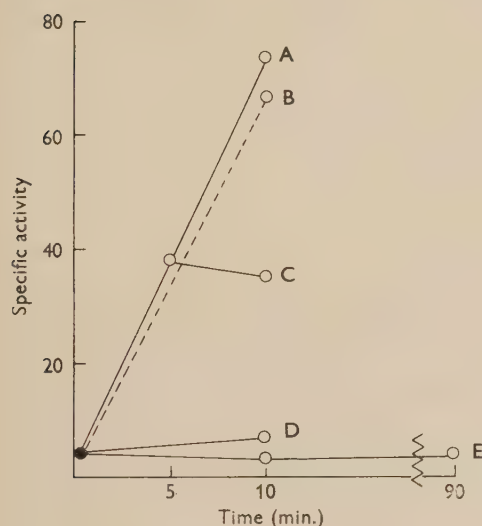


Fig. 3

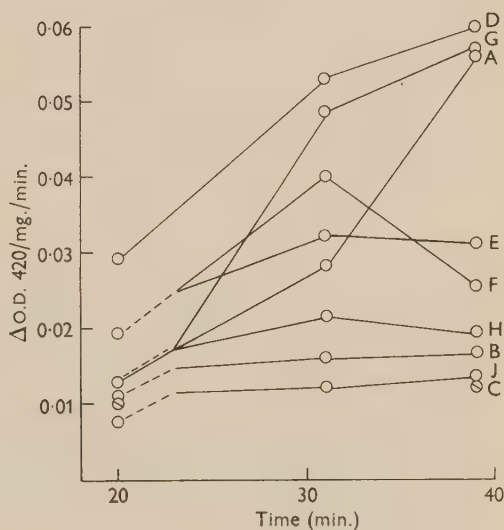


Fig. 4

Fig. 3. The effect of Mg⁺⁺, spermine and phage T2r⁺ on aspartate carbamyl transferase formation in *Escherichia coli* B, strain B₃₉. Curves: A = Mg⁺⁺, 0.01 M; B = A + spermine 10⁻³ M, at zero time; C = A with phage T2r⁺ added at 5 min.; D = B with phage T2r⁺ added at zero time; E = A with phage T2r⁺ added at zero time. The multiplicities of infection were 14.2 in curve C and 17.7 in curves D and E.

Fig. 4. Formation of alkaline phosphatase by *Escherichia coli* B, strain B₃₉.

Series 1. Multiplicity of infection = 35.8; no addition at zero time. Curve A, no addition; curve B, phage T2r⁺ added; curve C, phosphate added, two points only, ⊙.

Series 2. Multiplicity of infection = 25.1; uracil added at zero time. Curve D, no addition; curve E, phage T2r⁺ added; curve F, phosphate added.

Series 3. Multiplicity of infection = 32.6; no addition at zero time. Curve G, uracil added; curve H, uracil + phage T2r⁺ added; curve J, phosphate + uracil added.

Additions were made at 23 min. The final concentration of phosphate was 80 μmole/ml.; of uracil was 50 μg./ml.

Alkaline phosphatase

The synthesis of alkaline phosphatase was examined as another example and also because it is controlled by a different repressor, namely inorganic phosphate (Torriani, 1960). When organisms of *Escherichia coli* B, strain B₃₉, were suspended in a medium which did not contain inorganic phosphate, it was found that the presence or

absence of uracil did not markedly affect ability to make alkaline phosphatase enzyme (Fig. 4, curves A, D, G). The addition of inorganic phosphate (curves C, F, J) or phage T2r⁺ (curves B, E, H) caused inhibition of enzyme formation. It was found also that ghosts of phage T2r⁺ caused an inhibition of enzyme formation.

Table 2. *Dihydroorotic dehydrogenase activity in extracts from uninfected and infected cells of Escherichia coli B, strain B₃₉*

Organisms were suspended in basal medium with the additions noted in the table. The multiplicities of infection were 14·2 in C and 17·7 in B and E.

Additions	Time of addition of T2r ⁺ (min.)	Time of sampling (min.)	Orotic acid formed (μmole/mg./hr.)
A. Mg ⁺⁺ , 10 ⁻² M	—	0	5·3
		5	7·9
		10	11·8
B. Mg ⁺⁺ , 10 ⁻² M	0	10	4·5
C. Mg ⁺⁺ , 10 ⁻² M	5	10	6·7
D. Mg ⁺⁺ , 10 ⁻² M + spermine, 10 ⁻³ M	—	10	11·7
E. Mg ⁺⁺ , 10 ⁻² M + spermine, 10 ⁻³ M	0	10	4·1

DISCUSSION

Our results show that following infection with phage T2r⁺ or with phage ghosts, organisms of *Escherichia coli* B, strain B₃₉, did not synthesize certain enzymes, where the formation of the enzymes had been repressed by previous growth conditions, even though the repressors had been removed from the medium shortly before the infection. That the action of the phage in stopping the formation of host protein was due to a more favourable competition of the infecting system for available amino acids seems unlikely in view of the findings obtained here that supplementation of the infecting medium with amino acids did not prevent the inhibition of enzyme formation. A medium of greater osmotic pressure which might have decreased the loss of cell constituents into the medium also did not affect the inhibition of enzyme formation caused by phage infection. Although in some of the experiments presented, very small increases were seen in enzyme activity over those at the time of addition of the phage, these increased activities represented maximum values of 0·5–3 min. of continued synthesis based on the rate of synthesis of uninfected cells. The techniques used in the present work would not be able to detect whether synthesis actually continued after infection for these lengths of time, or whether there might have been a delay in the adsorption or penetration of phage, leading to small increases in the amount of enzyme.

The effects of ³²P decay on the viability of bacteria and on their ability to form enzymes have been interpreted as suggesting that intact deoxyribonucleic acid (DNA) is necessary for the synthesis of bacterial enzymes (McFall, Pardee & Stent, 1958; Riley, Pardee, Jacob & Monod, 1960). This hypothesis would provide a plausible explanation of the results observed here, since there is extensive breakdown of the host DNA following phage infection (Weed & Cohen, 1951; Kozloff, Knowlton, Putnam & Evans, 1951; Hershey, Dixon & Chase, 1953; Burton, 1955). In another situation, McFall & Magasanik (1960) found that alkaline phosphatase was formed in a thymine-deficient culture of *Escherichia coli*, strain 15T⁻, when the synthesis of

β -galactosidase and other proteins was inhibited. McFall & Magasanik suggested that the inhibition of β -galactosidase synthesis was a result, not of irreversible nuclear damage, but of repression. The possibility that the enzymes we have studied are not formed because of an accumulation of endogenous repressor substances seems to be unlikely, because the enzymes are involved in the synthesis of the precursors of DNA which are normally utilized by the phage-infected cell at an enhanced rate. However, other mechanisms are possible; one is suggested by the observations of the effect of phage infection on the permeability of the cell (Puck & Lee, 1954, 1955) and by the observation of Bessman & van Bibber (1959) that guanine deoxynucleotide kinase synthesized after phage infection requires different activating metal ions from the host enzyme which catalyses the same reaction. It is possible that the synthesis of host protein has different ionic requirements from those of the synthesis of protein in the phage infected cell.

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The Action of Tetanus Toxin in Frogs

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SUMMARY

The development of tetanus intoxication in the frog is dependent on the environmental temperature being above about 15°. The incubation period and time to death become shorter as the environmental temperature is raised. Cooling below about 15° prevents both the fixation of the toxin and its action after fixation but does not increase the speed at which the toxin is destroyed or excreted. The absorption of the toxin after injection into the dorsal lymph sac is not prevented by cooling. It is possible to produce local tetanus in frogs by the intramuscular injection of tetanus toxin, but the dose needed for this is very critical. In most cases either generalized tetanus develops or there are no signs of intoxication. However, local tetanus can regularly be produced when the toxin is given intramuscularly to frogs partially protected from generalized tetanus by an injection of tetanus antitoxin given via the dorsal lymph sac. The local tetanus so produced does not progress to involve the opposite limb. The muscular spasm is abolished by cutting the motor nerve or by general anaesthesia. Tetanus toxin appears to act on the central nervous system of the frog in the same manner as in mammals. However, frog brain tissue does not neutralize tetanus toxin in low concentrations as does mammalian brain tissue, but concentrated tetanus toxoid gives immediate protection to frogs in the same way as it does in mammals.

INTRODUCTION

The action of tetanus toxin on cold blooded vertebrates has not been extensively studied, but the majority of those species on which the toxin has been tested have proved susceptible. However, the dose of toxin required to produce tetanus is much greater than for even the most resistant of mammals and susceptibility is dependent on the environmental temperature being above a certain level. Courmont & Doyon (1893) were the first to observe that frogs became susceptible to tetanus toxin when kept at a temperature above 20°. Morgenroth (1900) endeavoured to analyse the mechanism of the temperature-dependent resistance of the frog to tetanus toxin. He inoculated frogs kept at 8° with tetanus toxin and found that they remained normal unless warmed to 32° when, after 2–3 days, they developed tetanus. However when, after being warmed for only 24 hr. and before signs of intoxication occurred, they were returned to the cold they remained in good health. On being warmed a second time, these frogs developed tetanus after a shorter interval than frogs similarly inoculated and warmed for the first time. Cold therefore arrests the development of tetanus even when part of the incubation process has taken place. It does not however annul the process of intoxication, because the sum of the two successive incubation periods remained the same as that of the single one which had been uninterrupted

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by any cooling. The only other amphibian about which the action of tetanus toxin has been reported is the axolotl, a larval salamander of the genus *Amblystoma*, which Metchnikoff (1897) found to be very sensitive. Of other poikilothermic vertebrates, there is one report on the effect of tetanus toxin on fish, by Lapenta (1932) who suggested that the gold fish *Carassius auratus* would be very suitable for the assay of tetanus toxin. The reptiles have been a little more fully investigated. Grasset & Zutendyk (1931), in South Africa, were able regularly to produce signs of tetanus in lizards, young crocodiles and snakes, but tortoises were only susceptible to intracerebrally injected toxin. Cowles & Nelson (1947) used the American crested lizard and were able to produce signs of tetanus. However, Metchnikoff (1897) failed to demonstrate tetanus in the European green lizard. With all the susceptible reptiles it was found that the development of tetanus intoxication depended on the environmental temperature. At low temperatures signs of tetanus either failed to appear or the incubation period was longer than at higher temperatures.

The present investigation was undertaken to see whether tetanus toxin appears to act in frogs in the same way as in mammals, or whether in these cold-blooded partially resistant animals it has some different mode of action.

METHODS

Frogs and aquaria. The frogs used in all the following experiments were of the common British species *Rana temporaria* and were obtained at intervals as required from Cornwall. They were of both sexes and varied in weight between 15 and 30 g. Galvanized iron tanks about 2 ft. square were used as aquaria. Different environmental temperatures were obtained by means of thermostatically-controlled low temperature electric immersion heaters or by placing the tanks in a refrigerated room. In most experiments control animals were kept in the same tank as the intoxicated ones. It was rare for a control frog to die from natural causes; they remained healthy over long periods at temperatures between 5° and 27°.

Blood samples from frogs. These were obtained by exposing the heart under ether anaesthesia and puncturing the ventricle with a fine glass pipette containing 10 mm.³ heparin (Liquemin, Roche Products Ltd, 5000 i.u./ml.). The blood was centrifuged and about 0.25 ml. plasma obtained per frog.

Tetanus toxin. The toxin preparation was kept as a finely ground powder in a vacuum desiccator until dissolved in sterile saline a few minutes before use. Two preparations of tetanus toxin were used. The first was some of that described by Wright, Morgan & Wright (1950) and will be referred to as toxin A; the LD₅₀ dose for mice, determined by the death time method of Ipsen (1941) was about 0.05 µg. The second preparation of tetanus toxin, toxin B, was provided by Dr Mollie Barr (The Wellcome Research Laboratories); the LD₅₀ dose for mice was 0.005 µg.

Tetanus antitoxin. The antitoxin containing 1500 international units/ml. was supplied by Burroughs Wellcome and Co. Ltd.

Tetanus toxoid. A concentrated preparation of tetanus toxoid (TD 343 D) containing 1175 Lf doses/ml. was provided by Dr Mollie Barr.

RESULTS

Generalized tetanus in frogs

The concentration of toxin in the blood following inoculation. In most of the following experiments the inoculations were made into the dorsal lymph sac. Thorotrast (a thorium dioxide preparation) injected by this route reaches the blood stream very rapidly and then slowly passes from the blood into the tissue fluids (Foxon & Rowson, 1956). That tetanus toxin behaves in the same way seemed very probable but some experiments were made to confirm this and to determine the effect of different environmental temperatures on the concentration of toxin in the blood following injection into the dorsal lymph sac. As it is not possible to take samples of blood from the same frog at intervals, a group of similar frogs were each injected with 1.0 mg. tetanus toxin A in 0.5 ml. saline and bled at different times. The frogs were kept in three groups at 5°, 15° and 26°.

Table 1. *The plasma concentration of tetanus toxin following injection into frog dorsal lymph sac*

Time after injection of 1.0 mg. tetanus toxin (days)	Frogs kept at		
	5°	15°	26°
	Plasma level of tetanus toxin (in mouse LD ₅₀ /ml.)		
0.125	—	560	625
1	185 (2)	520	450
2	280 (2)	235	300
3	305 (2)	130	340 (2)
4	310	230	275
5	295	150 (2)	250
6	300	—	350 (2)
8	150	—	—
12	—	85	—
14	190	—	—
18	—	62	—
19	—	26	—
21	150	—	—
33	250	—	—

Values marked (2) are the average of two experiments.

Table 1 shows the amount of tetanus toxin found in the plasma of these three groups of frogs. At 15° and 26° the blood concentration was very high after only 3 hr. and then decreased fairly rapidly during the next 3–4 days as the toxin passed from the plasma into other tissues. At 5° the concentration in the blood increased slowly during the first 3 days, to reach about the same value as that found in the other two groups at this time. The frogs at 5° were lethargic, showed very little voluntary movement even when disturbed, and the lymph hearts beat at a decreased rate. Therefore it was not surprising that toxin injected into the dorsal lymph sac reached the blood stream more slowly in frogs at 5° than in those at higher temperatures. Because the toxin was entering the circulation at a diminished rate in frogs at 5°, there was time for it to become distributed throughout the body fluids while it was being absorbed from the dorsal lymph sac, with the result that the maximum concentration in the blood found in these frogs was not so high as in those kept at 15° or

26°. In the frogs at 26° death occurred before the seventh day, so the toxin concentration could only be followed for 6 days. However, in the other two groups clinical tetanus did not develop and the blood toxin concentration was followed and found to decrease very slowly, especially in the frogs kept at 5°, where even after 33 days there was a high proportion of toxin still circulating. At 15° the amount of toxin in the plasma decreased more rapidly and after 19 days had reached such a low value that the frogs could be warmed to 26° without developing tetanus.

From this experiment it is clear that tetanus toxin rapidly reaches the general circulation from the dorsal lymph sac, and that although cooling slows down the rate of entry it does not prevent the toxin reaching the blood stream. The lower maximum blood toxin concentration in the frogs at 5° cannot account for their insusceptibility to tetanus since the frogs at 18° were also unaffected, and their blood toxin concentration was initially as high as in the group at 26° which developed tetanus.

The physical signs of generalized tetanus. After the injection of tetanus toxin into the dorsal lymph sac Gumprecht (1895) found that the onset of tetanus was generalized, but Brunner (1894) reported that the onset was in the fore limbs even when the toxin was injected into a back leg. Zupnik (1905) produced ascending tetanus by an inoculation into the dorsum of the foot but Fröhlich & Meyer (1916) were only able to obtain this effect by inoculation into the cord. In the present experiments, the injection of tetanus toxin into the dorsal lymph sac produced a generalized condition in which all four limbs were affected at the same time. In the very early stages there was only a slight limitation of movement, most easily seen in the hind legs when the frog was raised from the water by a hand placed under the forepart of the body. A normal frog easily climbs onto the hand, whereas a frog with early tetanus cannot bring its hind legs far enough forward; there was a limitation of flexion. Soon the fore legs became fixed across the front of the chest (Pl. 1, fig. 1) and the frog could swim, able to make ever decreasing movements with his hind legs, which hung down in the water; eventually the hind legs became rigidly extended (Pl. 1, fig. 2) and the frog floated helplessly on the water. Reflex spasms occurred when the condition was well developed but were only obvious in the hind legs. The lymph hearts did not seem to be affected by tetanus toxin and continued to beat normally up to the end. It is in fact only by their continued beating in the final stages that the presence of life can be discerned.

The rate at which the intoxication progressed depended on the body temperature. At 26° death often occurred within 24 hr. of the first signs of tetanus, whereas at 18° the signs slowly progressed for 3 to 4 days and a frog might remain completely unable to move for a day or two before death. However, although cooling slowed its rate of progress it did not annul the process.

The relationship of dose of toxin and body temperature to time of death. Table 2 shows the median death time for groups of frogs, all kept at 26° but injected with different doses of tetanus toxin A into the dorsal lymph sac. The death time rapidly increased when the dose of toxin was below 0.5 mg. The figures in Table 2 give a curve very similar to that obtained by Ipsen (1941) with mice, except that the LD 50 dose for a frog is very much larger and must be in the region of 150 μ g. as compared with 0.05 μ g. for a mouse. Therefore at 26° the frog is about 3000 times as resistant as the mouse, but in the frog the lethal dose differs very much with the body temperature.

The effect of body temperature on the death time can be seen in Table 3, where the

median death time is shown for groups of frogs kept at different environmental temperatures but each injected with 0.5 mg. tetanus toxin A into the dorsal lymph sac. Below 20° the death time rapidly increased; at about 18° the frogs appeared to become completely resistant to the action of tetanus toxin in that dosage. Probably if the dose of toxin were increased, tetanus would develop in the frogs kept at a slightly lower temperature than 20°, but it is clear that cooling had a pronounced protective action. This was particularly remarkable in view of the fact that the frogs remained

Table 2. *The relationship between toxin dosage and death time at 26°*

Groups of four frogs were used at each dose value. Toxin injected into dorsal lymph sac.

Dose of toxin (mg.)	Median death time (days)
2	6.5
1	7
0.5	8.5
0.25	16

Table 3. *The relationship between body temperature and death time*

Groups of 10 frogs were used at each temperature. Tetanus toxin A (0.5 mg.) injected in dorsal lymph sac.

Temperature (° C.)	Median death times (days)
28	3.0
23	7.5
18	Over 21

Table 4. *The effect of antitoxin on the death time of frogs at 27° when given at different intervals after a standard dose of toxin*

Groups of 6 frogs were used. Dose of antitoxin: 0.2 ml. 300 international units.
Dose of toxin: 0.5 mg. tetanus toxin A.

Interval between toxin and antitoxin (hr.)	Median death time (days)
24	Survived
48	8
72	4.5

quite active at temperatures down to 14° and only showed slight lethargy at 10°. The protective effect of a decrease in body temperature does not seem to be due to an alteration in the absorption or fate of the toxin in the body. Therefore the effect of environmental temperature on the fixation of toxin to, and action on, susceptible structures was studied.

Body temperature and the fixation of tetanus toxin. When tetanus toxin has passed to its site of action in the central nervous system and the development of tetanus cannot be prevented by the injection of antitoxin, it is said to have become fixed. In frogs living at 27° a lethal dose of toxin was fixed 48 hr. after injection as shown in Table 4.

This shows the results of an experiment in which three groups of frogs received a gross immunological excess of tetanus antitoxin (0.2 ml.) at different intervals after the injection of a lethal dose of tetanus toxin (0.5 mg. toxin A). By the end of 48 hr. although a lethal dose of tetanus toxin had been fixed the antitoxin was able to prolong the frog's life. Presumably this was because further toxin would have been fixed during the next 24 hr. After 3 days antitoxin has no effect on the death time. In contrast to the rapid fixation of tetanus toxin which took place at 27° was the complete failure of fixation at temperatures below about 18°. Frogs living at 15° were completely protected by antitoxin given a week or more after the inoculation of tetanus toxin, showing that fixation of a lethal dose had not occurred.

Table 5. *The effect of cooling from 26° to 18° for different periods beginning 48 hr. after the injection of tetanus toxin.*

Dose of tetanus toxin A: 1.0 mg.			
Number of frogs	Duration of cooling (days)	Median death time (days)	Increase in median death time due to cooling (days)
7	Not cooled	5.5	—
7	3	8.5	3
6	6	10.5	5
6	9	13.0	7.5

Table 6. *The effect of cooling from 26° to 5° for different periods beginning 48 hr. after the injection of tetanus toxin*

Dose of tetanus toxin A: 1.0 mg.			
Number of frogs	Duration of cooling (days)	Median death time (days)	Increase in median death time due to cooling (days)
6	Not cooled	7	—
6	6	14	7
6	9	16.5	9.5

Body temperature and the injurious action of tetanus toxin after fixation. From the observations recorded above it can be seen that cooling prevented the development of tetanus in the frogs and that the fixation of toxin was delayed and might not take place to the extent of a lethal dose. The action of toxin after fixation also seemed to be inhibited; this was shown by experiments in which groups of frogs were cooled after toxin had been allowed time to become fixed. All the frogs were inoculated with tetanus toxin (1.0 mg. toxin A) and kept at 26° for 48 hr. in order to ensure that a lethal dose of toxin had become fixed. In the first experiment, batches of these frogs were then transferred to a tank at 18° for different periods of time, at the end of which they were returned to the tank at 26°. Table 5 shows the length of time each group spent in the tank at 18° and their median death time. In column 4 of Table 5 the increase in the survival time over that of the control frogs is recorded, and it is clear that although the death time had been much prolonged by the cooling the increase was not quite equal to the duration of cooling. Therefore it appears that at 18° the

toxin was able to act, but only very slowly. In the second experiment frogs were cooled to 5° instead of to 18°. This more severe chilling prevented completely the injurious activity of the toxin (Table 6). The death time appeared to have been increased more than the duration of cooling (Table 6, column 4). If the action of the toxin was in fact annulled at this low temperature the increase in death time after cooling for 9 days ought to have been more than after 6 days, but it was not.

The effect of tetanus toxin given by different routes

The intramuscular route compared with inoculation into the dorsal lymph sac. It has been shown by several workers (Fildes, 1931, p. 308) that in mammals the minimal lethal dose of tetanus toxin by intravenous injection is about 5 times greater than the minimal lethal dose by intramuscular injection. An experiment was made to see whether this phenomenon could be shown in the frog. An inoculation of toxin via the dorsal lymph sac has been shown to be virtually equivalent to an intravenous injection, and so the effect of intramuscularly injected toxin was compared with the effect

Table 7. *The effect of tetanus toxin B given to groups of four frogs at 25° by different routes and in different dosages*

Dose of toxin B (mg.)	Median death time after inoculation into the	
	Dorsal lymph sac	Right gastrocnemius muscle
0.1	5 days	5 days
0.05	6 days	7 days
0.025	11 days	12 days
0.005	18 days	3 developed local tetanus
0.0025	Remained normal	Remained normal
0.0005	Remained normal	Remained normal
	Median death time after intracerebral injection	
0.01	2.5 days	
0.001	3.5 days	
0.0001	Remained normal	

of a similar dose given via the dorsal lymph sac. Two groups of 24 frogs were inoculated with tetanus toxin B, one group intramuscularly in the right gastrocnemius muscle, and the other via the dorsal lymph sac. Each group was divided into six subgroups, which received doses of toxin as shown in Table 7. The volume of toxin solution given to each frog was the same (0.1 ml.) and they were all kept at 25° after inoculation. With 0.025 mg. or greater doses of tetanus toxin B there was no significant difference in the median death time or in the signs of tetanus produced in the two groups; the frogs all died of generalized tetanus. However, with 0.005 mg. toxin B three of the four frogs inoculated intramuscularly developed a mild local tetanus in the inoculated limb. They were able to swim but there was a marked reduction of flexion at the knee, and to some extent at the hip, as compared with the normal leg. The opposite leg was not involved and the condition did not progress or regress during the 21 days for which these frogs were observed.

From Table 7 it appears that the minimal lethal dose of tetanus toxin was slightly larger by intramuscular injection than by inoculation via the dorsal lymph sac. This is the reverse of what has been described in mammals and may be due to the toxin

escaping from frog muscle more quickly than from mammalian muscle because of the greater blood capillary permeability in frogs. This view is supported by the failure to produce local tetanus by intramuscular injection of toxin, when the dose was more than 0.005 mg. With a large dose of toxin most of it escapes from the muscle but some passes up the motor nerve. The toxin reaches the central nervous system by two routes and this must be a less lethal process than when all the toxin is injected into the dorsal lymph sac. With a small intramuscular dose of toxin the amount which escapes is not enough to give generalized tetanus and the amount of toxin going up the nerve is only enough to produce a very mild local tetanus.

Intracerebral inoculation. With mammals tetanus toxin has always been found most lethal and dramatic in its action when injected into the central nervous system; this proved to be so with the frogs studied here. Frogs were anaesthetized with ether and 0.01 ml. toxin solution injected through the skull into the brain with a 31-gauge needle. From Table 7 it is clear that the dose of toxin required to kill was very much smaller when it was given intracerebrally than by either of the other two routes, and the death time for a given dose of toxin was much shorter. Cooling to 16° prevented the development of tetanus after an intracerebral injection of toxin. Two groups of three frogs were inoculated intracerebrally with 0.01 mg. tetanus toxin A and kept at 16°. After 6 days no signs of tetanus were observed. One group was then moved to a tank at 25° where they developed generalized tetanus after 24 hr. The second group was moved to the warm tank after 11 days in the cold and then developed tetanus after a further 24 hr. Tetanus following the intracerebral inoculation of toxin was very rapid in its progress and a frog which appeared normal in the morning might show gross signs of generalized tetanus in a few hours and die in the evening. In some cases after the intracerebral inoculation of tetanus toxin frogs showed signs of irritability and on being disturbed moved violently around the tank in a quite abnormal manner.

Intraneural inoculation of the sciatic nerve. In mammals the injection of a very small dose of toxin into a motor nerve will produce a severe local tetanus in the muscles supplied by the inoculated nerve. Some experiments were made to produce local tetanus in the frog by this technique. Under ether anaesthesia the sciatic nerve was exposed through a vertical incision in the back of the thigh. A piece of sterile filter paper was placed under the nerve and 0.01 ml. solution containing 0.2 mg. tetanus toxin A was injected with a 31-gauge needle. The frog's sciatic nerve is a very delicate structure and the amount of toxin which remained in the nerve was questionable as a large part appeared to leak onto the filter paper. The incision was closed and the frogs kept in a tank at 26°. In no case did local tetanus develop. A few frogs developed generalized tetanus, no doubt due to toxin which leaked from the nerve entering the general circulation. The intraneural inoculation could not be considered as satisfactory and no conclusions can be drawn from the results.

The production of local tetanus

It is possible to produce local tetanus consistently in the frog by the intramuscular injection of tetanus toxin. However the dosage is critical and the tetanus mild, for if too much toxin is given generalized tetanus develops as the first sign of intoxication. This is probably due to toxin leaking from the injection site into the general circulation. The local tetanus which follows the intramuscular injection of a suitable dose of

toxin is so lacking in severity that it may not be recognized and in a group of frogs similarly inoculated only a proportion will show local tetanus. Having observed that a slight local tetanus could be produced by intramuscularly injected toxin it seemed probable that with a larger dose of toxin a more severe local tetanus might be masked by the generalized tetanus which developed from toxin which had leaked into the general circulation. Therefore it was decided to try to neutralize toxin which entered the general circulation by giving tetanus antitoxin via the dorsal lymph sac a few minutes before the intramuscular injection of a large dose of toxin.

Table 8. *The effect of intramuscularly injected tetanus toxin in frogs protected by various doses of antitoxin in the general circulation. Similar doses of toxin and antitoxin were given to mice*

Dose of tetanus toxin B: 0.2 mg. in 0.05 ml. saline.

Dose of antitoxin (units)	Fate of frogs	Fate of mice
94	No intoxication	No intoxication
23	No intoxication	No intoxication
6	Local tetanus	No intoxication
1.5	Generalized tetanus	Generalized tetanus
0.4	Generalized tetanus	Generalized tetanus

Five pairs of frogs were given different doses of tetanus antitoxin, as shown in Table 8. Fifteen minutes later they were each inoculated with the same dose of tetanus toxin B (0.2 mg./0.05 ml. saline) into the right gastrocnemius muscle and put in a tank at 25°. The same quantities of toxin and antitoxin were mixed and injected intravenously into mice to determine whether or not antitoxin was in excess. The frogs which received 94 and 23 units of antitoxin were completely protected. However the pair given 6 units developed a well-marked local tetanus (see Pl. 1, fig. 3). The local tetanus was first apparent on the fifth day after injection of the toxin and did not significantly alter during the next 16 days. The mice which received 6 units of antitoxin survived. The smaller doses of antitoxin which were used did not protect and the mice and the frogs developed generalized tetanus.

In another experiment 10 frogs were given 6 units of tetanus antitoxin into the dorsal lymph sac and 15 min. later they were divided into two groups. One group received each 0.2 mg. tetanus toxin B in 0.05 ml. of solution intraperitoneally; the other group received the same dose of toxin into the right gastrocnemius muscle. The five frogs given toxin intraperitoneally showed no signs of intoxication during the 21 days for which they were observed, whereas 3 of the 5 frogs inoculated intramuscularly developed a marked local tetanus on the seventh day. Although the local tetanus was moderately severe there was no ascending involvement of the central nervous system and the opposite limb never showed any signs of tetanus.

General anaesthesia with ether abolished the muscular spasm in the affected limb and both legs became equally flaccid. On recovery from the anaesthetic the spasm returned. Two of the frogs with local tetanus were anaesthetized and the sciatic nerve, in the affected limb, cut. This produced a permanently flaccid limb, the spasm of local tetanus not returning on recovery from the anaesthetic. Local tetanus in the frog is therefore dependent, as in mammals, on an intact motor nerve supply and an unanaesthetized central nervous system.

It was suggested (Wright, 1953) that the force behind the neural transport of substances is the pressure produced inside contracting voluntary muscles. Frogs do not exhibit those frequent movements, typical of mammals. They will sit for long periods completely motionless and it seemed possible that the centripetal neural transport of tetanus toxin might only occur when the intramuscular pressure was raised during active movement. The toxin would therefore pass centrally more slowly in the frog than in mammals and would have a greater opportunity to escape from the muscle into which it had been injected. This would account for the rather poor development of local tetanus in the frog as compared with other laboratory animals.

Table 9. *The effect of exercise on the development of tetanus in frogs given 0.02 mg. toxin B and kept at 25°*

Group	Day of experiment on which death occurred
Exercise	6, 6, 7, 7, 7.
No exercise	6, 7, 7, 7, 8.

The above hypothesis was tested in two experiments. In the first, 8 frogs were given 0.005 mg. tetanus toxin B in 0.05 ml. solution, intramuscularly in the right gastrocnemius muscle. They were then divided into two groups; one group was put in a dark tank and not disturbed; the other group was placed in a tank of deep water where they had to keep swimming. Both groups were kept at 25° and after 24 hr. the 8 frogs were put together in one tank. On the eighth day of the experiment the frogs in both groups began to develop local tetanus which did not progress beyond the inoculated limb and was not more severe in one group than the other. In the second experiment a larger dose of tetanus toxin B was used (0.02 mg. in 0.05 ml. solution) and the group of frogs given exercise were kept swimming and hopping for 6 hr. before the two groups were put together in one tank at 25°. Again there was no difference between the two groups in survival time and none of the frogs showed local tetanus (Table 9).

The inactivation of tetanus toxin with brain tissue. The excised mammalian brain and spinal cord are capable of combining with large quantities of tetanus toxin; in doing so they inactivate the toxin (see Fildes, 1931, for an account of the Wassermann-Takaki reaction). The grey matter of the brain is more active than the white matter or the spinal cord in this reaction; no other body tissue shows any comparable neutralizing effect. The brains of birds and cold-blooded animals were found not to neutralize tetanus toxin by Metchnikoff (1898) and it was suggested by Knorr (1897) that their high degree of resistance to tetanus might be connected with the inability of the nervous tissue to combine with the toxin. The fact that many cold-blooded animals are susceptible to tetanus when warmed suggested that either there is a change in the combining power of the brain tissue on warming or that the phenomenon is unconnected with the development of tetanus.

The effect of mixing brain tissue with tetanus toxin was investigated by taking two equal samples of tetanus toxin, each dissolved in 1.0 ml. of saline and adding mashed brain tissue to one and an equal volume of saline to the other, which acted as a control. Penicillin (50,000 units) was added to each tube to prevent bacterial growth. After a period of incubation, the amount of free tetanus toxin in each specimen was then determined by inoculating mice intravenously with 0.2 ml. of the supernatant

fluid from each tube. In the first experiment 0.5 g. of brain tissue was placed in contact with 0.1 mg. tetanus toxin A at room temperature (18°) for 17 hr. Under these conditions mouse brain neutralized 90 % of the toxin, whereas with frog brain no neutralization could be demonstrated. Even when tetanus toxin + frog brain were incubated together at 26° or 37° no neutralization appeared to take place. The possibility that brain tissue from a frog kept at 26° for some days would react differently was examined. Three frogs were kept at 26° for 7 days before their brain tissue was removed and incubated as before with tetanus toxin at 26°. Again no neutralization was observed, although at this temperature the intact animals would have been susceptible to tetanus.

Another theory to explain the failure of frog brain tissue to neutralize tetanus toxin was that some accessory factor, not present in frog brain tissue, is required for the fixation of tetanus toxin. This hypothetical substance might be present in mammalian blood and would be mixed with mammalian brain brei in its preparation. This hypothesis was tested in two experiments. In the first, fresh rabbit serum was mixed with frog brain tissue and the mixture tested as above, no neutralization of tetanus toxin was demonstrated. In the second experiment the possibility that peritoneal fluid might contain the factor was tested by inoculating 3 mice intraperitoneally with a mixture containing 5 mouse LD₅₀ doses of tetanus toxin and 0.5 g. frog brain tissue. These mice developed tetanus and died, showing no increase in survival time as compared with control mice which received toxin only.

The specific precocious protective action of tetanus toxoid

The specific precocious protective action of tetanus toxoid first reported by Krech (1949) is probably due to toxoid molecules competing with toxin molecules for some receptor substance (Davies & Wright, 1955). As brain tissue from frogs cannot be induced to neutralize tetanus toxin it must be in some way different from that of mammals. It was therefore of interest to know whether tetanus toxoid would have any protective action in frogs. To demonstrate this phenomenon it is necessary to have a concentrated toxoid preparation; the one used contained 1175 Lf/ml. Seven

Table 10. *The protective action of tetanus toxoid in frogs at 25°*

Time of toxoid injection before the toxin (hr.)	No. of frogs	Period of survival after injection of toxin (days)
No toxoid	7	5, 6, 6, 6, 6, 6, 6.
$\frac{1}{4}$	4	10, 12, 15, 15.
24	3	11, 11, 16.

frogs were inoculated with 1.0 ml. of this concentrated toxoid via the dorsal lymph sac and placed with 7 uninoculated control frogs in a tank at 25°. After 15 min. the 7 controls and 4 of the previously inoculated frogs were each given 0.1 mg. tetanus toxin B via the dorsal lymph sac. On the next day (24 hr. after receiving the tetanus toxoid) the 3 remaining test frogs were given a similar dose of toxin. Table 10 shows the survival time of the three groups of frogs in this experiment. It can be seen that the

concentrated tetanus toxoid very considerably prolonged the survival time of the frogs but did not prevent the eventual development of tetanus. Tetanus toxoid seems to have the same protective action in the frog as in mammals.

DISCUSSION

Frogs can be kept at environmental temperatures between 4° and 28° but below about 10° they become noticeably lethargic and at 4° they remain motionless and only respond sluggishly to external stimuli. Above about 15° they are sensitive to the action of tetanus toxin but below this temperature they become resistant, although they remain active and respond readily to any disturbance. The change in sensitivity to tetanus toxin comes before there is any noticeable alteration in behaviour and before there is any demonstrable slowing in the rate of absorption of substances injected into the dorsal lymph sac. It seems clear from the experiments on the blood tetanus toxin concentration following its injection into the dorsal lymph sac, that the protective action of cooling is not due to a failure to absorb the toxin into the general circulation. These experiments also demonstrate that the toxin is circulating in an active form and has not been neutralized or toxoided by the frog tissues.

Tetanus toxin produces the same picture of unco-ordinated muscular contraction in the frog as in mammals, and the rate at which the intoxication progresses depends on the dose of toxin. However when using frogs in place of mammals there is an important additional dimension of experimental freedom: the environmental temperature. This might be of value for the analysis of the mode of action of the toxin. Cooling by a few degrees prevents both the fixation of the toxin and its action after fixation but it does not reverse either process. This protective effect of a low environmental temperature must be due either to an alteration in the frog's metabolism or to the toxin being able to act only above a certain temperature. It seems most unlikely that a change in body temperature which does not affect the normal activity of a frog could be accompanied by an alteration in neuromuscular physiology sufficiently profound to give complete resistance to tetanus toxin. It seems more probable that the effect of temperature is on the action of the toxin. Tetanus toxin is a protein and is active at a molecular concentration much below that required for most poisons. This suggests that it may be enzymic in nature and if so, below a certain temperature its combination with substrate and subsequent action might be so slow as to have virtually no effect. If this be true, mammals should also become resistant to tetanus toxin below a certain temperature. There are two reports which suggest that the environmental temperature has some slight effect on the resistance of mammals to tetanus toxin. A seasonal variation in the susceptibility of guinea pigs was reported by Herwick, Weir & Tatum (1936); they found that the lethal dose of tetanus toxin was 0.006 mg./kg. body weight in winter and 0.004 mg./kg. in summer. The observations were made over a period of two years and the authors pointed out that any change in the potency of the toxin would probably be a slow deterioration, whereas they found it to retain the same strength at the same season a year later. The increased resistance of these guinea pigs to tetanus toxin in the winter might be due to some cyclic physiological change not directly due to the environmental temperature. Ipsen (1951) observed a similar phenomenon in mice during hot and cold periods and made experiments in which mice were maintained continuously at environmental temperatures of 10°, 25°

and 35°. Different doses of tetanus toxin were tested and it was found that at 10° the survival time was generally longer than normal while at 35° it was shorter. Unfortunately in neither of these two series of experiments do we know the actual body temperature of the animals.

In mammals the site of action of tetanus toxin has been identified as a blocking of inhibitory hyperpolarization of the neurones which control the motor activities of the spinal cord (Brooks, Curtis & Eccles, 1957). In the frog the following evidence also points to a central site of action: (1) the dose of toxin required to produce generalized tetanus is smaller and the incubation period shorter when given intracerebrally than by other routes; (2) it is difficult to produce local tetanus in the frog by intramuscular injection of toxin, suggesting that it does not act on the nerve endings or muscles; (3) local tetanus is dependent on an intact motor nerve supply, which is at least consistent with the view that the toxin acts centrally.

Although tetanus toxin appears to have the same action in frogs as in mammals, the frog at its most sensitive is about 3000 times as resistant as a mouse. This might be due to a relatively impermeable blood brain barrier, but the frog is not very susceptible even to intracerebrally injected toxin and the ratio of lethal dose via the dorsal lymph sac to lethal dose intracerebrally is only about ten, a figure smaller than that reported for some mammals (Wright, 1955, p. 440). Therefore the frog's resistance must be due either to an insusceptible brain substance or to the fact that the toxin is operating at a temperature some 10° lower in the frog than in the mouse. If frogs could be kept at 37° they would almost certainly be more sensitive to the action of tetanus toxin than at 26°, but probably they would not be as susceptible as mice, for there must be other factors involved. It is relevant that birds, which have a high body temperature, are very resistant to tetanus toxin irrespective of the route by which it is given (Davies, Morgan & Wright, 1955).

It is not easy to produce local tetanus in frogs. This is probably partly due to toxin being washed from the site of injection by the very great lymph production in the frog's tissues (Isayma, 1924) and partly to the relatively high concentration of toxin required in the central nervous system to produce signs of intoxication. However, when the frogs are partially protected by antitoxin given via the dorsal lymph sac, a moderately severe local tetanus follows intramuscularly injected toxin. Circulating antitoxin will also protect them against a dose of toxin given intraperitoneally, but not against the same dose of toxin intramuscularly. A similar experiment was reported by Friedemann, Zuger & Hollander (1939) with guinea pigs. They found that when a dose of toxin was given intramuscularly it required between 5 and 8 times as much antitoxin to protect the guinea pigs as would have been necessary had the same dose of toxin been given intravenously. The development of local tetanus in frogs protected against circulating toxin is good evidence for the neural transport of tetanus toxin in the frog. Unfortunately the conclusive experiments involving nerve sclerosis (Baylis, Mackintosh, Morgan & Wright, 1952) and localized anaesthesia (Wright, Morgan & Wright, 1952) which have been applied to the rabbit to prove the central site of action of tetanus toxin in local tetanus, cannot for technical reasons be used to elucidate the pathology of local tetanus in the frog.

It has not proved possible to demonstrate any neutralization of tetanus toxin by frog brain tissue, using low concentrations of toxin, and in this frogs may resemble birds. Metchnikoff (1898) was unable to demonstrate the neutralization of tetanus toxin by

bird brain tissue, but recently van Heyningen (1959*a*) found chicken brain to be as active as mammalian brain tissue in the absorption of tetanus toxin. The reaction between brain tissue and tetanus toxin is a reversible absorption process (Fulthorpe, 1956), the amount of toxin absorbed depending on the concentration of toxin. Metchnikoff may well have failed to demonstrate absorption of the toxin by bird brain because he used too low a concentration of toxin. The active substance in brain tissue has recently been identified (van Heyningen, 1959*b*) as a ganglioside and it seems probable that the combination of the toxin with this substance may be the first step in the action of the toxin. If this be so the high resistance of birds and cold-blooded vertebrates to tetanus toxin might be due to their brain tissue not combining with low concentrations of the toxin.

I wish to thank Professor G. Payling Wright for his help and advice in the preparation of this paper.

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Fig. 1

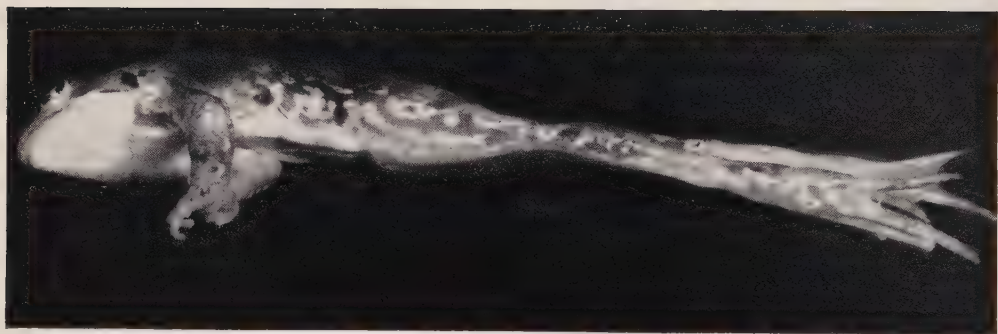


Fig. 2



Fig. 3

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EXPLANATION OF PLATE 1

- Fig. 1. A frog with generalized tetanus showing the fore legs fixed across the front of the chest.
- Fig. 2. The same frog as in Fig. 1 showing the extreme extension of the back legs.
- Fig. 3. Two frogs showing local tetanus of the right leg.

The Characterization of *Pseudomonas fluorescens* with the Aid of an Electronic Computer

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SUMMARY

Data which had been used to characterize the species *Pseudomonas fluorescens* (Rhodes, 1959), as distinct from the data used to define the genus *Pseudomonas*, have now been used to obtain an assessment of the over-all similarity of 169 isolates of polarly flagellate bacteria (mainly of the *P. fluorescens* species-group) by means of an electronic computer. The results of this analysis are compared with the arrangement of isolates previously favoured by the author. It was found that the two arrangements were often closely similar; the discrepancies are discussed. The two aeromonads of the collection were picked out by the computer, which also satisfactorily replaced an accidentally misplaced pseudomonad isolate. One hundred and thirty-four isolates previously considered to belong to the *P. fluorescens* species-group were positioned together by the computer in a group with more than 80.0% over-all similarity throughout. This confirmed previous conclusions about the validity of this taxonomic unit, even though the description of it may appear imprecise because each character was not possessed by every isolate. The possibility of further species differentiation within this group is discussed, particularly with regard to *P. aeruginosa*. The results suggest that *P. aeruginosa* may be regarded as a variety of *P. fluorescens*. Other evidence was obtained which suggests that further work on the phytopathogenic pseudomonads may well reveal significant differences from *P. fluorescens*. Factors governing the choice of a type strain or culture are discussed.

INTRODUCTION

A study of 169 isolates of polarly flagellate bacteria was described by Rhodes (1959), and suggestions were made for a revised definition of the genus *Pseudomonas* on the basis of 26 cytological, cultural and biochemical characters common to 165 of the isolates. A further 43 characters were not common to these 165 isolates, and the author discussed whether these characters might be used to define subgroups (? species), although it was evident that very few of the isolates were identical with respect to all these 43 characters. From the examination of this body of data, many attempts were made to discover any subgroups of related isolates. Certain of the characters (e.g. gelatin digestion, milk digestion, nitrate reduction, aesculin hydrolysis, egg-yolk reaction, ability to grow at 5°) which have been considered to be useful criteria for distinguishing species in *Pseudomonas* and closely related genera (e.g. *Chromobacterium*; Sneath, 1956) were selected as possible differentiating criteria. It was found that there was no complete correlation between any of the 43 characters under consideration (nos. 1-43 listed in Fig. 1). The resultant sub-

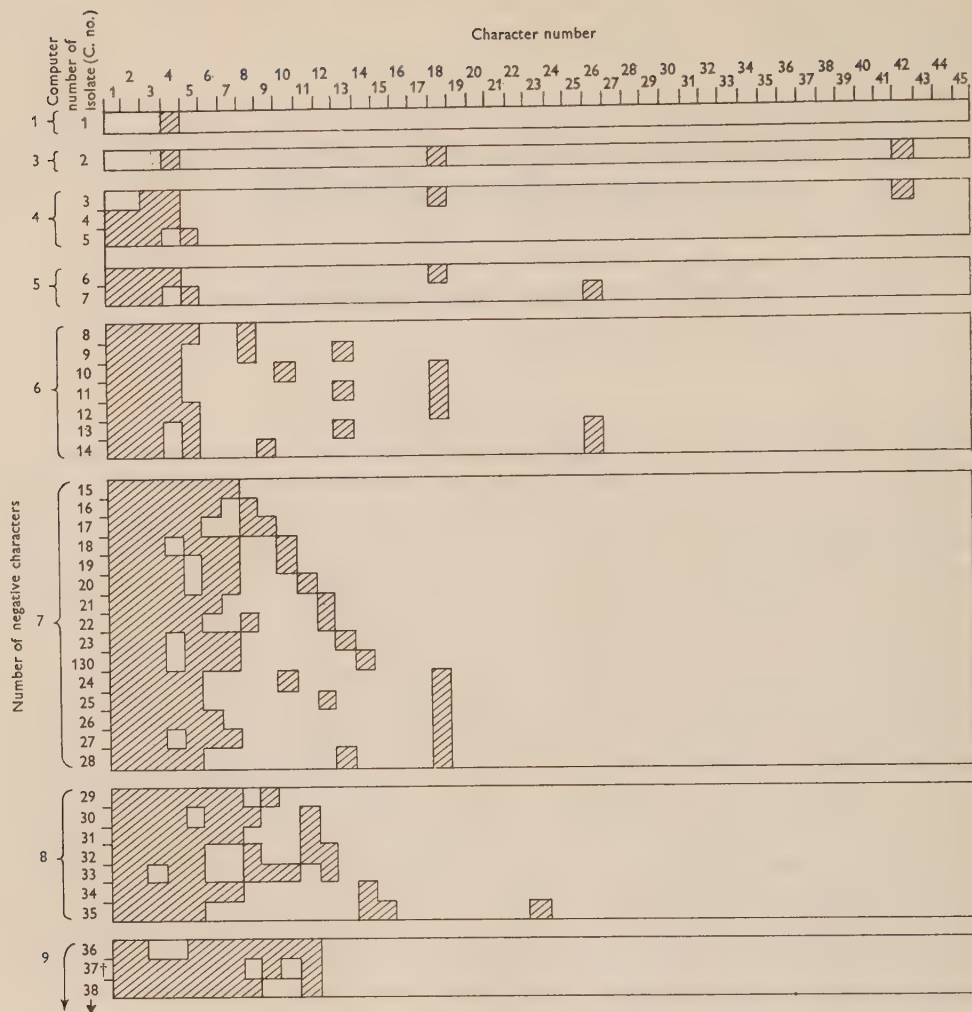


Fig. 1. The properties of the 39 most reactive isolates of *Pseudomonas* and their arrangement by the author in a table constructed to demonstrate over-all similarity according to the numerical principles described in the text. Hatched areas indicate negative test results; clear areas indicate positive test results. † = proposed neotype strain of *Pseudomonas fluorescens* (NCTC no. 10,038; ATCC no. 13,525) selected by Rhodes (1959). Key to character numbers: 1 = H_2S from Kligler's medium; 2 = growth at 42° ; 3 = H_2S from cystine; 4 = aesculin hydrolysis; 5 = growth in 6.5 % NaCl broth; 6 = nitrite reduction; 7 = nitrate reduction; 8 = growth from raffinose-C; 9 = growth in minus-C control; 10 = growth in minus-N control; 11 = growth from urea-N; 12 = growth from sucrose-C; 13 = hydrolysis of 'Tween 80'; 14 = milk digestion; 15 = egg-yolk reaction; 16 = olive oil hydrolysis; 17 = growth from inositol-C; 18 = H_2S from thiosulphate; 19 = growth from cellobiose-C; 20 = tributyrin hydrolysis; 21 = gluconic acid oxidation; 22 = growth from trehalose-C; 23 = gelatin liquefaction; 24 = growth from rhamnose-C; 25 = growth in bile-salt broth; 26 = growth from D-arabinose-C; 27 = growth in 5.0 % NaCl broth; 28 = growth from succinate-C; 29 = growth from acetate-C; 30 = growth from mannitol-C; 31 = growth from lactic acid-C; 32 = growth from lactose-C; 33 = growth from glycerol-C; 34 = growth from xylose-C; 35 = growth from formate-C; 36 = growth from L-arabinose-C; 37 = growth from maltose-C; 38 = growth at pH 5.0; 39 = growth from glucose-C; 40 = growth from galactose-C; 41 = growth at 5° ; 42 = growth from fructose-C; 43 = growth from L-malic acid-C; 44 = growth from citrate-C; 45 = growth from sorbitol-C.

groups had no other characters in common and therefore were not considered to be useful taxonomic units. The fact that some of these characters had also been shown sometimes to be unstable supported this decision. Three interpretations of this situation were considered. (a) Among the 165 isolates, 39 were replicates; the remaining 126 isolates might be representatives of 126 different subgroups, each differing in at least one of the 43 characters. (b) Perhaps all the 165 isolates belonged to one group with 26 characters in common, with a further 43 characters which might be either present or absent. To adopt this interpretation would be controversial, at least because of the inclusion of about 20 named phytopathogenic species as well as *Pseudomonas aeruginosa* in this grouping. (c) Perhaps further subdivisions were possible only on the basis of an estimate of the over-all resemblance of the isolates when compared with each other with reference to these 43 characters.

The taxonomic method of 'classification from below' (White, 1937) involves the study of isolates as fully as possible, estimation of the degree of similarity between them, and a final arrangement of the isolates so that similar ones are closely grouped, a greater separation of the isolates (or groups of very similar isolates) being directly related to the magnitude of their dissimilarity. This method was discussed and preferred by Rhodes (1956, 1959) as also was the final adoption of the Adansonian principle of according an equal weight to each character studied. The author constructed a table on the basis of a numerical estimate of the over-all similarity of the 165 isolates. This was done by counting the number of positive or negative test results obtained for each of the 43 characters studied. The isolates were then arranged in a sequence such that the first isolate was the most reactive (with 42/43 positive test characters), and the last isolate was the least reactive (only 4/43 positive test characters). The other isolates were then arranged between these extremes (2nd to 164th positions) in order of gradually decreasing reactivity. For example, the 2nd, 3rd and 4th places in the sequence were occupied by isolates of which each had 40 positive test characters; the 5th, 6th and 7th places, 39; the 8th, 9th and 10th places, 38; etc. It became obvious that this ordering of isolates could be split up into subgroups in which each isolate of a given subgroup had the same number of positive characters. However, such subgroups were not found to be useful taxonomic units, because seldom were the same characters negative in any one subgroup (see Fig. 1). Therefore an attempt was made to order the isolates within each of the subgroups. For this, each character was considered separately. In the collection as a whole the frequency of the possession of a positive reaction for each character was useful information, and could be used to determine the order of the isolates in each subgroup in the following way. The % of all isolates which showed a positive test result for a given character was calculated for each character. The characters were then arranged in an order of increasing % value. The characters were then numbered serially (1-43); this was the character order number (in brief, character number). For example:

Character number: 1, 2, 3, 4, to 43

Isolates positive (%): 1, 4, 12, 17, to 99

The final order of the isolates in each subgroup was then determined on the principle of according priority within each subgroup to those characters which were more

frequently negative. For example, subgroup E might contain 3 isolates each with 4 negative characters as follows:

	Position of isolate	Character number									
		1	2	3	4	5	6	7	8	9	10 to 43
Subgroup E	10th	+	-	-	+	-	+	+	-	+	All +
	11th	+	+	-	-	-	+	+	-	+	All +
	12th	+	+	+	-	-	+	+	-	-	All +

The final order of isolates in the above example was chosen to be 10, 11, 12 because, when the frequency of positive reactions in the collection as a whole was considered, the characters 2 and 3 were more frequently negative than characters 4 and 5. In this way isolates with negative reactions which were relatively more common were given a smaller ordinal number in the sequence than those with negative reactions which were less common. This minimized the weight of a given negative reaction the more frequently it occurred. This was a deviation from the Adansonian principle of according equal weight to each character, because an isolate with a more unusual negative character would be placed in a relatively later position in each subgroup than if the given character were not so weighted. This arrangement was the culmination of many attempts to give an equal taxonomic weight to each test character. The full table cannot be reproduced here; part of it is given in Fig. 1 and shows how strains may be compared and their similarity illustrated. Fig. 1 also shows the overlapping relationships between isolates.

It appeared that the 165 isolates arranged as just discussed formed a closely related series which showed gradually decreasing reactivity when expressed on the basis of numbers of positive characters. No clear divisions were apparent, but many phytopathogens were concentrated at the 'less-reactive' end of the table and *Pseudomonas aeruginosa* isolates were at the 'reactive' end.

It was clear that the above table contained a body of data suitable for analysis by means of an electronic computer as used by Sneath (1957*a, b*) to estimate the over-all similarity on Adansonian principles. With the kind assistance of Dr P. H. A. Sneath the complete set of data was analysed by Elliott Brothers Ltd., by use of the Elliott 405 digital computer and the programme 'Bacterial Classification Mark I' as outlined by Sneath (1957*b*). Only 130 isolates were analysed, but these represented the whole collection (including 4 non-*Pseudomonas* isolates) because the remaining 39 isolates were duplicates of some of the 130 analysed. Table 1 lists the code numbers of the isolates as given by Rhodes (1959) together with the new code numbers needed by the computer. For comparative purposes the position of the 165 isolates in the author's complete table mentioned above (used in Rhodes, 1959) is also included. For the computation 45 characters were used, namely the 43 characters already discussed and named in Fig. 1, together with the results for citrate and sorbitol utilization. The scoring of the features for the computer was simplified because, for theoretical reasons, no quantitative distinctions were made; each character tested for was scored as either positive or negative.

A 'non-coincidental triangle' table (S table) of similarity (S) values for each pair of isolates with respect to the 45 features was obtained after electronic computation, according to the formula

$$S = \frac{n_s}{n_s + n_d}$$

Table 1. (a) *The code numbers of the Pseudomonas isolates studied by Rhodes (1959); (b) the position of the isolates in the author's table constructed to show the relationships between the 169 isolates when they were compared with respect to 45 test characters (see text for details); (c) the new computer code number (C. no.)*

Parentheses around numbers indicate that the isolates enclosed in those positions had identical properties, and so only one new computer code number was necessary. (†) = proposed neotype strain *Pseudomonas fluorescens* (Rhodes, 1959); (*) = NCTC *P. fluorescens*; (C) = *P. chlororaphis*; (A) = *P. aeruginosa*; (S) = *P. synchyanea*; (P) = phytopathogenic *Pseudomonas* species; (Ae) = *Aeromonas*; (X) = *Xanthomonas*; (N) = non-pseudomonad phytopathogen.

Isolate code number	Position in author's complete table	Computer number (C. no.)	Isolate code number	Position in author's complete table	Computer number (C. no.)
5940 (*)	1st	1	25/1; d244 (P)	(96, 97th)	66
20, 21, 24/2 (A)	(2, 3, 4)	2	52/9	98	67
24/1 (A)	5	3	8/6	99	68
36/4	6	4	9/1	100	69
d289 (P)	7	5	49	101	70
8/11	8	6	27/6	102	71
47/1, 47/4	(9, 10)	7	10/4	103	72
22/5, 26/4, 36/3	(11, 12, 13)	8	5/3	104	73
10/3	14	9	4/3, 12/4, 38	(105, 106, 107)	74
36/1	15	10	52/6	108	75
22/6	16	11	40/2	109	76
22/1, 22/4, 36/2	(17, 18, 19)	12	32, 34, 43/2, 43/3,	(110-114)	77
47/3, 47/5	(20, 21)	13	KBI		
47/2	22	14	d251 (P)	115	78
52/1, 52/5; d52 (P)	(23, 24, 25)	15	53/1	116	79
46	26	16	41	117	80
4/2, 15/6	(27, 28)	17	5/10	118	81
52/4	29	18	14/1, 27/2	(119, 120)	82
35/1	30	19	56/1	121	83
8/3, 8/14, 26/5, 50	(31-34)	20	12/5, 27/5	(122, 123)	84
d53 (P)	35	21	5/5	124	85
26/3, 54	(36, 37)	22	52/10	125	86
8/4	38	23	27/3	126	87
13/3	40	24	15/4	127	88
31/3	41	25	19	128	89
56/3	42	26	5/2	129	90
12/1, 12/6	(43, 44)	27	9/3	130	91
26/2, 45	(45, 46)	28	56/4	131	92
28/6, 52/2	(47, 48)	29	d152 (P)	132	93
7/3, 8/2, 8/15;	(49-52)	30	10/1	133	94
d263 (P)			7810 (Ae)	134	95
8/5, 8/7, 8/13, 26/1,	(53-57)	31	13/1	135	96
28/4			15/5	136	97
8/9	58	32	27/4	137	98
51	59	33	9/4	138	99
52/8	60	34	43/1	139	100
8/12	61	35	12/3	140	101
23/3	62	36	31/1, 31/2	(141, 142)	102
28/3; 28/5 (†)	(63, 64)	37	5/8	143	103
8/16	65	38	56/2	144	104
27/1	66	39	18/3	145	105
53/2	67	40	40/3	146	106
8/8	68	41	15/1	147	107
22/3	69	42	44	148	108
7357 (C)	70	43	43/4	149	109
8049 (Ae)	71	44	13/4	150	110
23/4	72	45	42/3	151	111
52/3	73	46	9/2	152	112
7/6	74	47	23/1	153	113
5/1	75	48	d221 (P)	154	114
16/1	76	49	18/2	155	115
8/10	77	50	42/2	156	116
30	78	51	d236 (P)	157	117
42/1	79	52	d281 (P)	158	118
23/5	80	53	d270 (P)	159	119
5/4	81	54	3246 (S)	160	120
15/3	82	55	d133 (P)	161	121
55	83	56	25/2 (X)	162	122
26/6	84	57	d248 (P)	163	123
3	85	58	d72 (P)	164	124
39	86	59	d260 (P)	165	125
22/2, 22/7	(87, 88)	60	d300 (P)	166	126
16/2	89	61	d69 (P)	167	127
13/2, 14/2	(90, 91)	62	d64 (P)	168	128
40/1	92	63	d73 (N)	169	129
35/2, 35/3	(93, 94)	64	8/1	39	130
52/7	95	65			

as used by Sneath (1957*b*), where n_s is the number of positive features possessed by both isolates, and n_d is the number of positive features possessed by the first isolate but not by the second + the number of features possessed by the second isolate but not by the first.

A sorting of the isolates into groups at 75.0, 80.0, 85.0, 90.0 and 95.0% similarity levels of association (L levels) was also made by the computer. At the 75.0% level only 16/130 isolates remained ungrouped; these were sorted by visual inspection of the S table. Then family trees were constructed in an attempt to show the inter-relationships revealed by the analysis.

RESULTS AND DISCUSSION

The order of the arrangement of isolates made by the computer was compared with the order which resulted when the numerical principles described above were used. Figure 1 illustrates a section of the experimental data and Table 2 gives a sample of the arrangements of the author (Rhodes, 1959) and the computer. In the sequel, C. no. = computer code number. The following conclusions may be drawn.

(a) The two arrangements of the isolates were often in close agreement (see Table 2).

Table 2. *A comparison of the arrangements of the 39 most reactive isolates of Pseudomonas as made by the author and the computer*

C. no. = Computer code number. † = proposed neotype strain of *Pseudomonas fluorescens* (NCTC no. 10,038; ATCC no. 13,525) selected by Rhodes (1959). * = isolates for which the author's placement and the computer's placement differed by more than 10 places.

Position in author's table†	Order of isolates as arranged by the		Position in author's table	Order of isolates as arranged by the	
	Author (C. no.)	Computer (C. no.)		Author (C. no.)	Computer (C. no.)
1	1	1	21	21	25
2	2	2	22	22	26
3	3	3	23	23*	28
4	4	4	24	130*	13
5	5	6	25	24	14
6	6	10	26	25	29
7	7	11	27	26	30
8	8	12	28	27	31
9	9	5	29	28	32
10	10	7	30	29	34
11	11	8	31	30	35
12	12	9	32	31	27
13	13*	15	33	32	43
14	14*	16	34	33*	18
15	15	17	35	34	23
16	16	19	36	35	130
17	17	20	37	36*	38
18	18*	21	38	37†	39
19	19	22	39	38	37†
20	20	24			

† Rhodes (1959).

(b) Of the 7 isolates illustrated in Table 2 which showed positions differing by more than 10 places, for 5 of them (C. no. 13, 14, 18, 23, 130) this was apparently due to the author's failure to give sufficient weight to an isolate which possessed a relatively rare character (aesculin hydrolysis). For example, isolate C. no. 23 which was aesculin positive and had only 7 negative characters was computed to be 12 places later in the sequence and after isolate C. no. 35 which had 8 negative characters. Similarly, isolate C. no. 36 with two relatively rare positive characters (growth at 42°; ability to produce H₂S from cystine) was placed much later (in the 77th position) by the computer. Likewise isolate C. no. 33 which possessed three relatively rare positive characters and only 8 negative ones was given a position 14 places later by the computer than by the author, and then came after isolates with 9 or even 10 negative characters. Therefore the fact that my placements which were too near the 'reactive' beginning of my table in comparison with the order given by the computer, suggests that the possession of even one relatively rare positive character did not receive sufficient emphasis as a dissimilarity when a comparison was made on my numerical basis. In other words, the formula used by Sneath (1957*b*) for the electronic computation gave a better over-all comparison than was achieved solely by numerical counting of numbers of positive and negative characters. This was because when using the latter method relatively rare positive characters were not sufficiently weighted as dissimilarities.

(c) The case of isolate C. no. 130 was consoling. This isolate was placed after isolate C. no. 23 in the author's original table (see Fig. 1; Tables 1 and 2) but in the preparation of the data for the computer it was accidentally omitted and added to the list as isolate C. no. 130 (see Table 1). The computer placed it back to follow isolate C. no. 23 once more.

(d) The only other gross aberrations in the comparison concerned isolate NCTC8049 which was placed 44th by the author and 108th by the computer, and isolate NCTC7810 which was placed 95th and 109th respectively. These isolates were the two aeromonads already differentiated from the rest of the collection by other criteria (Rhodes, 1959). They were included here to test the electronic computation method which indeed placed them together, even though one had only 9 and the other 15 negative characters. They were positioned by the computer between the *Pseudomonas fluorescens*-like isolates and the phytopathogens (see Ae, Fig. 2); but this positioning is misleading because the characters used to differentiate the genus *Aeromonas* were not among those considered in the present work (see later). Another non-*Pseudomonas* isolate (C. no. 129) was clearly differentiated from the rest by the computer (see Fig. 2) at the 15.0% level; the one xanthomonad was grouped with two other phytopathogens at the 45.0% level.

The S table was converted to a shaded diagram as suggested by Sneath (1957*b*). It was evident that the isolates down to C. no. 110 formed a large group with 80.0% over-all similarity throughout. The sorting procedure revealed that within this large group some subgroups with a higher % over-all similarity were present. These are shown more clearly in Fig. 2 than in the shaded diagram; it was not considered worth while to re-arrange the S table because of the high over-all similarity of all the isolates in the large group. The shaded diagram proved too large and complex to reproduce here; it has been photographed and copies are available.

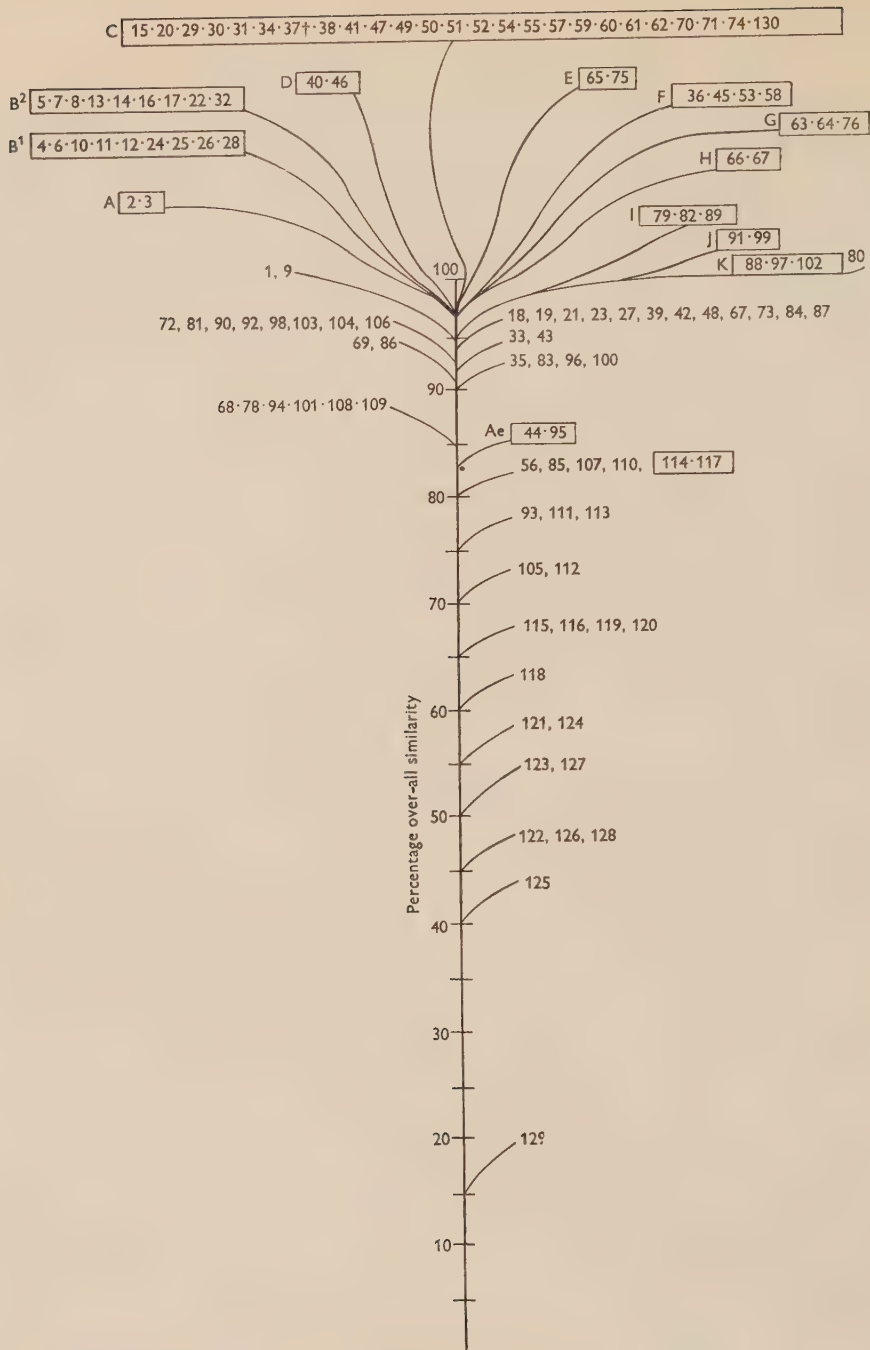


Fig. 2. Diagram showing the inter-relationships between 130 isolates, mainly *Pseudomonas*, constructed from the S table of over-all similarities and sorted by the electronic computer on the basis of the 45 test characters listed in Fig. 1.

Note that further isolates may be included in some of the groups, i.e. replicates listed in Table 1. Boxed-in groups of isolate numbers, indicated by letters, contain isolates with more than 97.0% over-all similarity among themselves. Numbers indicate the computer no. (C. no.) of the isolate. † = proposed neotype strain of *P. fluorescens* (Rhodes, 1959); group A = *P. aeruginosa* isolates; groups B to K together with isolates down to 110 (at 80.0% S level) = *P. fluorescens* species-group; between 40.0 and 70.0% S values = phytopathogenic *Pseudomonas* species; group Ae = *Aeromonas* isolates (but see text about 'true' position) 122 = ? *Xanthomonas* sp.; 129 = non-pseudomonad phytopathogen.

Thus the analysis by the electronic computer confirmed the previous conclusions of the author about the recognition of the 134 soil- and water-inhabiting pseudomonads as a group of closely related organisms. It also confirmed that the description of the species *Pseudomonas fluorescens* yielded a useful taxonomic unit, even though there were no absolute correlations between any two of the descriptive characters. Indeed, many of the characters were often absent from the isolates examined. This large group was known to contain isolates of *P. aeruginosa* and

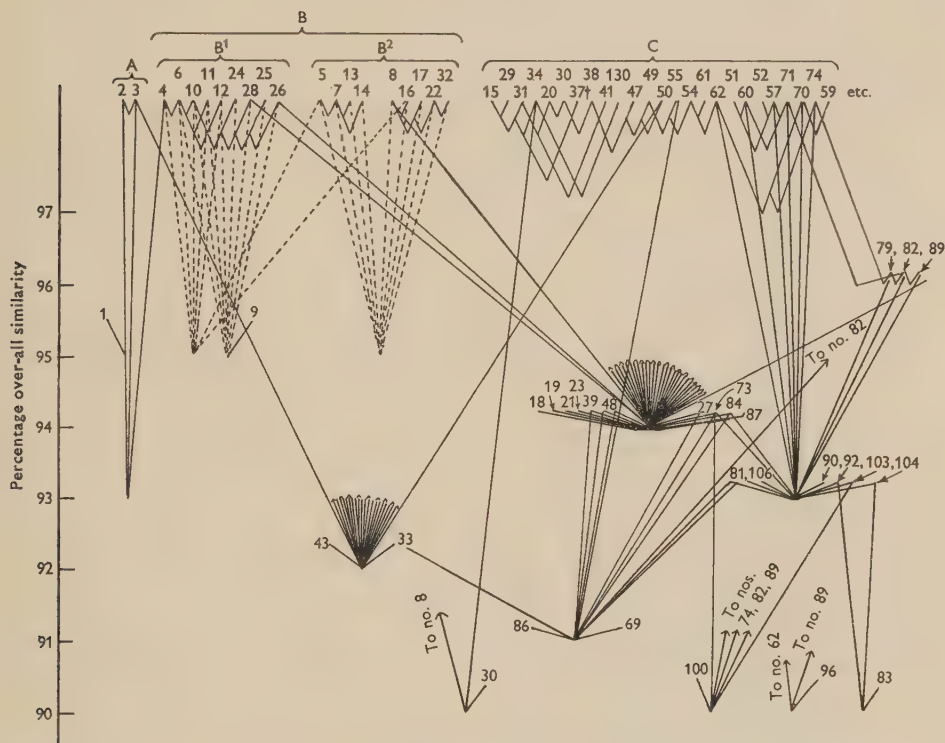


Fig. 3. Diagram showing the inter-relationships between many isolates of *Pseudomonas* when their over-all similarity was assessed on the basis of 45 test characters (listed in Fig. 1). The lines join isolates together at various levels of over-all similarity. It is apparent that a given isolate may show an equally close similarity with members in various groups, thus exhibiting multiple relationships. The dotted lines are used merely for clarity and are not otherwise significant. All numbers (some not included owing to the mechanical difficulties of reproduction) refer to the computer number (C. no.) of the isolate. † = proposed neotype strain of *Pseudomonas fluorescens* (Rhodes, 1959).

five named phytopathogenic species from the National Collection of Type Cultures. The close similarity of these with *P. fluorescens* has already been discussed (Rhodes, 1959). It may be relevant that the phytopathogens examined had been maintained in laboratory culture for a long time, and at least one of them (d52, *P. medicaginis* var. *phaseolicola*) when examined was found to be identical with two non-phytopathogenic isolates (see Table 1) and did not produce the characteristic greasy spots when inoculated into young bean pods. Nor was it susceptible to active *P. medicaginis* var. *phaseolicola* bacteriophage (Dr Eve Billing, personal communication).

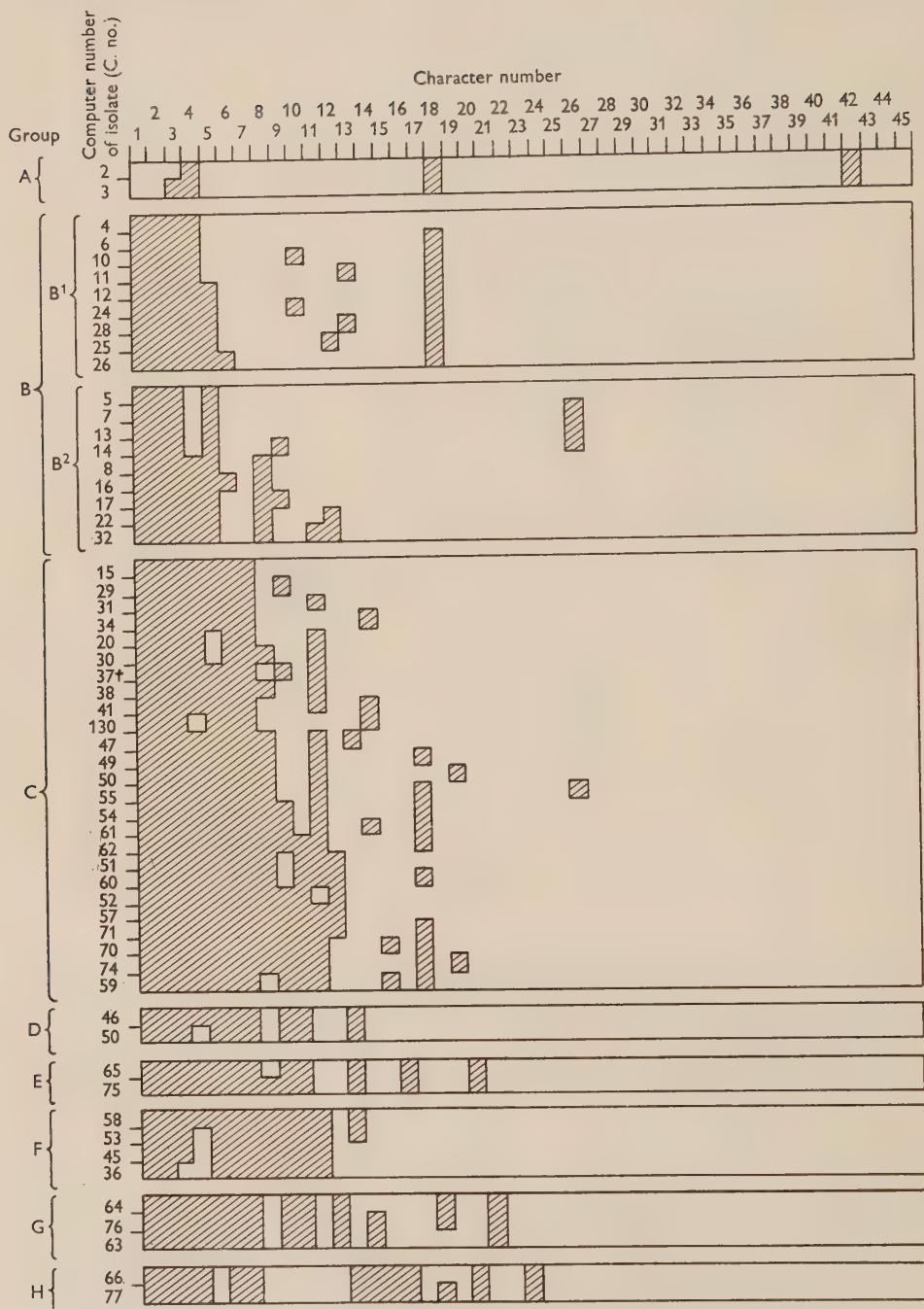


Fig. 4. For legend see opposite.

Figure 3 is another diagrammatic method for showing some of the more detailed relationships revealed by the S table; it attempts to show the multiple and overlapping relationships between some of the isolates. It can be seen that some groups (labelled A, B, C, etc., in the diagrams) of more closely similar isolates could be differentiated within the large *Pseudomonas fluorescens* group. The question whether such subgroups merit the rank of species, subspecies or variety is a major problem of bacterial taxonomy (see discussion by Sneath, 1957*b*, pp. 212–216). If one wishes to retain *P. aeruginosa* as a species then, according to this analysis, this means that all the aeruginosa-like isolates (group A here) should have an overall similarity of more than about 95.0%, because at the 93.0% level group A (aeruginosa-type) and group B (fluorescens-type) are linked by isolates such as C. no. 4 (see Fig. 3) which is definitely fluorescens-like. Furthermore, at the 92.0% level aeruginosa-like isolate C. no. 3 has a similarity of 92.0% or more with at least 30 fluorescens-type isolates. Therefore if group A were to be regarded as a species (*P. aeruginosa*), then by the same standards (based on S values) groups B, C, D, E, F, G and H (see Figs. 2–4) should also be considered as species. Whether group B is itself composed of 2 or 3 species, i.e. groups B¹ and B² (see Figs. 2–4) is also debatable. To help the crystallization of opinion Fig. 4 is given to show the familiar bacteriological properties of each of these groups and the range of variation exhibited. It is apparent that black and white definitions of such groups (? species) are not possible. In the author's opinion not one of these groups merits species rank; they are better to be regarded as units of lower taxonomic rank, e.g. varieties of *P. fluorescens*, even though the group A isolates are typical *P. aeruginosa*. This conclusion is, of course, controversial. It raises taxonomic and nomenclatural difficulties, because *P. aeruginosa* was designated as the type species of the conserved genus *Pseudomonas* by the Judicial Commission of the International Committee on Bacteriological Nomenclature (1952). Nevertheless, it confirms the practical experiences of many *Pseudomonas* taxonomists (see Haynes, 1951; Gaby & Free, 1953; Gaby & Hadley, 1957) about the close similarity of *P. aeruginosa* and *P.*

Fig. 4. The bacteriological properties of isolates of *Pseudomonas* which had been grouped together by the electronic computer at an over-all similarity level of more than 97.0% on the basis of 45 test characters (listed below). Groups are indicated by capital letters and are the same as the groups shown in Fig. 2. Hatched areas indicate negative test results; clear areas indicate positive test results. † = proposed neotype strain of *Pseudomonas fluorescens* (Rhodes, 1959). Key to character numbers: 1 = H₂S from Kligler's medium; 2 = growth at 42°; 3 = H₂S from cystine; 4 = aesculin hydrolysis; 5 = growth in 6.5% NaCl-broth; 6 = nitrite reduction; 7 = nitrate reduction; 8 = growth from raffinose-C; 9 = growth in minus-C control; 10 = growth in minus-N control; 11 = growth in urea-N; 12 = growth from sucrose-C; 13 = hydrolysis of 'Tween-80'; 14 = milk digestion; 15 = egg-yolk reaction; 16 = olive oil hydrolysis; 17 = growth from inositol-C; 18 = H₂S from thiosulphate; 19 = growth from cellobiose-C; 20 = tributyrin hydrolysis; 21 = gluconic acid oxidation; 22 = growth from trehalose-C; 23 = gelatin liquefaction; 24 = growth from rhamnose-C; 25 = growth in bile-salt broth; 26 = growth from D-arabinose-C; 27 = growth in 5.0% NaCl-broth; 28 = growth from succinate-C; 29 = growth from acetate-C; 30 = growth from mannitol-C; 31 = growth from lactic acid-C; 32 = growth from lactose-C; 33 = growth from glycerol-C; 34 = growth from xylose-C; 35 = growth from formate-C; 36 = growth from L-arabinose-C; 37 = growth from maltose-C; 38 = growth at pH 5.0; 39 = growth from glucose-C; 40 = growth from galactose-C; 41 = growth at 5°; 42 = growth from fructose-C; 43 = growth from L-malic acid-C; 44 = growth from citrate-C; 45 = growth from sorbitol-C.

fluorescens. The difficulties encountered when attempts were made to define the 'highly dissociable' genotype species *P. aeruginosa* were described by Gaby (1955) who found it impossible to designate a type culture; no exact definition of it is available. The analogous situation with the anthrax bacillus may be mentioned and particularly the reasoning of Smith, Gordon & Clark (1952) which led to their conclusion that the anthrax bacillus was best to be regarded as a potentially pathogenic variety of *Bacillus cereus*. Evidence from the literature and practical experience support the hypothesis that *P. aeruginosa* may be a variety derived from a fluorescens-like wild-type organism as a result of adaptation to the specialized environment of the animal body with which it is usually associated directly or indirectly. *P. aeruginosa* seldom occurs elsewhere, perhaps because it cannot withstand a temperature of less than 5° for more than 3 months (when stored in nutrient broth culture; personal observation). Lethal cold-shocking of *P. aeruginosa* was recently described by Gorrill & McNeil (1960). Finally, in connexion with the question of species rank, the convincing *Chromobacterium* subgroups which were designated as species by Sneath (1957*b*) appeared as separate groups down to an S value of 64.0%; his % values are perhaps comparable with mine because the experimental criteria from which they were derived were similar.

The position of the two *Aeromonas* isolates and their apparent fusion with the *Pseudomonas fluorescens* group at the 83.0% S level (see Fig. 2) is misleading because their distinctly different non-pseudomonad properties, which relate to their characteristic fermentative metabolism, were characters not included in the present analysis. A comparison was made which included all the typical aeromonad properties (see Rhodes, 1959, p. 248) as well as those used in this analysis. The S level values were then 58.0 and 45.0% for *Aeromonas ichthyosmia* and *A. hydrophila*, respectively, when they were compared with the proposed neotype strain of *P. fluorescens*.

The 19 isolates at the lowest part of Fig. 2 were different from the rest of the collection, and in most cases also differed from each other. This agrees with the fact that they consisted of representatives of at least 11 named phytopathogens, *Pseudomonas syncyanea* and 2 non-*Pseudomonas* isolates. This suggests that differences which merit species rank may be revealed when larger numbers of phytopathogenic isolates are similarly studied.

Another method of demonstrating some of the S table data was suggested by Cheeseman & Berridge (1959). This involved the arrangement of the isolates in a histogram which showed the numbers of S values of more than 70.0% possessed by each isolate. This method was examined in the present work. The results are given in Fig. 5 (solid lines). The order given by this treatment of the data did not follow the computer order at all closely, except for the placement of the same last 25 isolates. Those isolates with a large number of high S values were presumed to be most closely interrelated and therefore most typical of the whole group (Cheeseman & Berridge, 1959). Such reasoning implies that the first isolate here, C. no. 4, with 82 S values of 70.0% would be a better neotype strain of *Pseudomonas fluorescens* than the isolate C. no. 37 previously selected by the author (deposited in the NCTC as No. 10,038 and in the American Type Culture Collection, ATCC, as No. 13,525). It was felt by the author (Rhodes, 1959) that the most useful type strain of a group of organisms showing a given range of +ve or -ve characters should occur centrally

within this range, as indeed C. no. 37 does. Isolate C. no. 4 is considered by me to be unusually reactive. I think that in practice the likelihood that an unidentified new *P. fluorescens*-like isolate would be closely similar to such a type strain would probably be less than if a mediumly reactive isolate such as isolate C. no. 37 were selected as type strain.

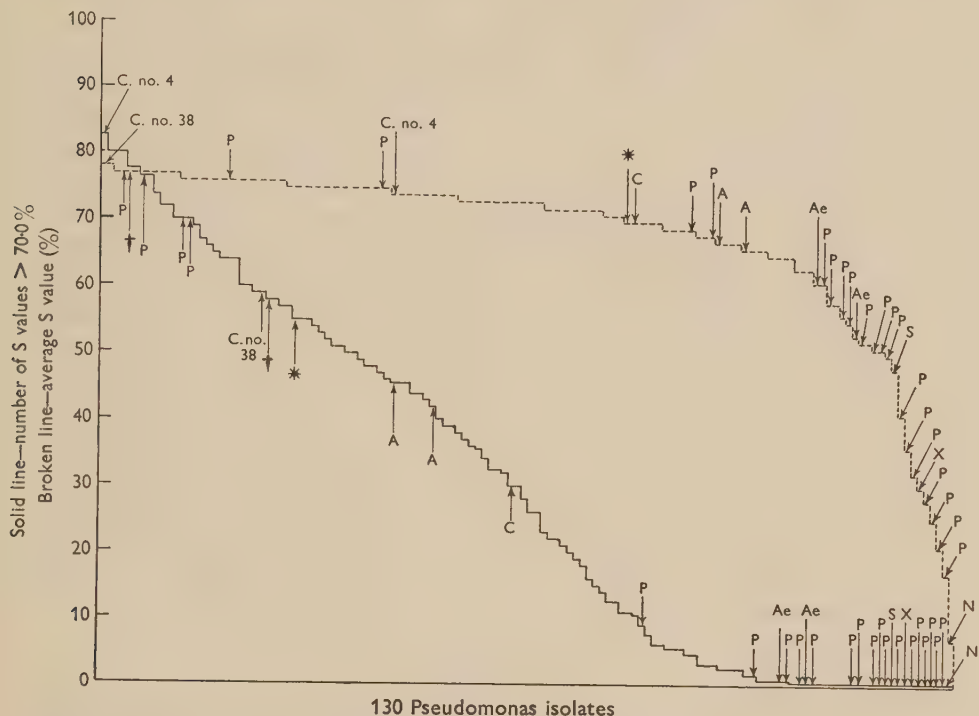


Fig. 5. Histograms showing the numbers of S values of more than 70.0% possessed by each of 130 isolates of (mainly) *Pseudomonas* (solid lines), and also the average S values of each isolate (broken lines). It is not possible to include the code numbers of each isolate in the diagrams, but some of the isolates discussed are shown by letters, the key for which is: † = proposed neotype strain *Pseudomonas fluorescens* (Rhodes, 1959); * = NCTC *P. fluorescens*; C = *P. chlororaphis*; A = *P. aeruginosa*; S = *P. syncyanea*; P = phytopathogenic *Pseudomonas* sp.; Ae = *Aeromonas* sp.; X = *Xanthomonas* sp.; N = non-pseudomonad phytopathogen; C. no. 4 = computer number 4 *P. fluorescens* isolate 36/4; C. no. 38 = computer number 38 *P. fluorescens* isolate 8/16.

I am grateful to Dr P. H. A. Sneath for pointing out that for a given isolate, average S values rather than numbers of S values of greater than 70.0% are perhaps better criteria for isolate comparison. Following this suggestion, the average S values for each of the 130 'computer' isolates were calculated by adding together all the appropriate rows and columns of numbers in the S table and dividing the total for each isolate by 129 (i.e. ignoring the 100.0% value of self-comparison). The results are shown in Fig. 5 (broken lines). The highest average S value was 78.11%, given by isolate C. no. 38; isolate C. no. 4 (discussed above) was relegated to the 45th position. In all the diagrams it is evident that isolate C. no. 38 is very similar to the proposed (Rhodes, 1959) neotype strain *Pseudomonas fluorescens* (isolate C. no. 37 here). Isolate C. no. 37 also has the 5th highest average S value

(77.84%). Therefore the suitability of isolate C. no. 37 to be the type strain of *P. fluorescens* is confirmed; it fulfils the requirements of a 'type culture' of a bacterial species as defined in *Bergey's Manual* (1957, p. 21) namely: 'A species of bacterium is the type culture or specimen together with all the other cultures or specimens regarded by an investigator to be sufficiently like the type (or sufficiently closely related to it) to be grouped with it.'

An inspection of the average S values of each isolate of the collection examined here showed a group of 152 isolates (including replicates; see Table 1) with average S values between 63.0 and 78.0%. The whole group included 7 phytopathogenic species of *Pseudomonas*, *P. chlororaphis*, 5 isolates of *P. aeruginosa*, 2 aeromonads, and 137 fluorescens-type isolates. Of these latter 137 isolates, 134 were exactly the same soil- and water-type isolates as were used for the suggested (Rhodes, 1959) revised description of *P. fluorescens* Migula, 1894. It is apparent that the analysis by the computer has confirmed many of the views held by the author (Rhodes, 1959) about the complex inter-relationships between pseudomonad isolates even when these were obtained only after the operation of the considerable selective factors inherent in the methods of isolation (e.g. one temperature, one medium, only fluorescent colonies selected), maintenance and study. The advantages of the judicious use of mechanical aids, such as an electronic computer in the analysis of data are obvious; but the tedium involved in the acquisition of sufficiently large quantities of reliable data under standardized conditions has not yet been circumvented. However, the author feels that the use of the electronic computer facilitates some advance from the situation criticized by Rahn (1929) who wrote: 'The method of attacking taxonomical problems by securing a large number of strains is the most hopeful of all ways; but the treatment of the data has not always shown that a real distinction has been made between species and strains.'

I am much indebted to Dr P. H. A. Sneath (National Institute for Medical Research, Mill Hill, London) for his interest and assistance, and to the Research Board of the University of Reading for a grant enabling the hire of the computer. Elliott Brothers Ltd. (Boreham Wood, Hertfordshire, England) most generously processed all my data, and I wish to thank Mr G. P. Wayne for his services on behalf of the firm. The interest and help of Professor B. C. J. G. Knight in the preparation of this paper is also gratefully acknowledged.

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Association of a Serum Opacity Reaction with Serological Type in *Streptococcus pyogenes*

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SUMMARY

Krumwiede (1954) reported that some group A streptococci formed a lipoproteinase which was capable of producing opalescence in horse serum. A survey of a large number of strains of group A streptococci has shown that those strains which carry an easily identifiable M antigen rarely produce the serum opalescence reaction, except for Types 2 and 9. On the other hand those types that elaborate M antigen poorly or not at all are usually good producers of the serum opacity reaction.

INTRODUCTION

In 1954 Krumwiede reported that some group A streptococci formed a lipoproteinase which produced opalescence in serum. Ward & Rudd (1938) had noted that the production of similar opalescence in serum broth was confined to a few of the Lancefield types of group A streptococci, although they tested only one strain of each type. The association of serological types of group A *Streptococcus pyogenes* with the production of particular enzyme systems has been the subject of numerous reports. Crowley (1944, 1954) noted this effect with respect to both hyaluronidase and amylase production. Robinson, Blinn & Frank (1952) and Williams (1954) reported similarly with glucuronidase and Lazarides & Bernheimer (1957) with diphosphopyridine nucleotidase. This paper reports a comparison of the type antigens and ability to produce an opalescence in serum broth of a large number of strains.

METHODS

Organisms. A collection of 732 strains of group A streptococci were examined; of these 344 were obtained from lyophilized stocks held in the Streptococcus Reference Laboratory. These strains, collected during the last 20 years or more from clinical material, included the original Griffith type strains. The remaining strains were those submitted for routine type identification during a period of about 6 weeks. All the latter strains were grouped and typed at the time of testing for the serum opacity reaction, while only a selected number of the stock strains were retested for type antigens.

Media. Serum broth consisting of one part Hartley broth (pancreatic digest of beef) and three parts horse serum, sterilized by filtration, was used for the demonstration of the serum opacity reaction; final pH 7.5 by glass electrode. Serum from normal horses was obtained from the Medical Research Council Serum Laboratories, Carshalton.

Proteinase was determined by the milk thioglycollate method of Elliott & Dole (1947).

Lipoproteinase. Urea extraction of the organisms and the demonstration in the extract of lipoproteinase activity by paper electrophoresis were carried out by the method reported by Krumwiede (1954).

Hyaluronidase. Two hyaluronidase preparations were used: a culture fluid from a hyaluronidase-producing group A streptococcus; a bovine testicular extract. These preparations were checked for activity by the method of di Ferrante (1956).

Serological grouping and typing of the organisms were done by the methods described by Williams (1958).

Serum opalescence. The ability of each organism to produce a serum opalescence was determined as follows. All strains were grown overnight at 37° in Todd-Hewitt broth to a final turbidity corresponding to about 10⁸ chains/ml. One loopful (0.01 ml.) of the culture was transferred to 3 ml. of the serum broth which was then incubated for 18–24 hr. at 37°. At this time the opalescence of the medium was noted and the growth tested for purity and viability by plating one loopful on a blood agar plate. No quantitative assessment of the opalescence was attempted; the results were scored as positive or negative. Different observers were responsible for the results reported for the stock and laboratory groups of strains respectively.

The observed reaction was the production of an opalescence (or increase in opacity) in the serum broth used. This opalescence probably occurs because certain serum components are rendered insoluble. The lipoproteinase described by Krumwiede was a specific enzyme attacking the α_1 lipoprotein of horse serum. The observable action of the lipoproteinase on horse serum is the production of a similar opalescence, but since many other factors might also produce this effect we have preferred the term serum opacity reaction, although we assume that the observed reaction is probably due to the lipoproteinase.

The results were not obscured by turbidity due to the growth of the streptococci, possibly because of the small difference between the refractive indices of the bacteria and of a medium containing 75 % (v/v) horse serum. Wichelhausen, Clark, Griffing & Robinson (1958) showed that it was possible to have 10⁷–10⁸ viable bacteria present in a 25 % (v/v) albumin solution without any visible turbidity.

RESULTS

The top half of Table 1 records the serum opacity reaction of stock and routine strains of various types. At the time the work was performed, the laboratory had available anti-M precipitin typing sera against streptococci of Types 1, 2, 3, 5, 6, 9, 12, 14, 15, 17, 18, 19, 23, 24, 26, 29, 30, 31, 33, 36, 37, 39, and 43. The lower half shows the results obtained with stock strains of other known types for which no anti-M sera were available. The strains had been assigned to these types at some time in the past on the basis of an M antigen. They are, however, 'difficult' types with which to prepare good anti-M precipitin sera. Table 2 gives the results found with strains identified by agglutination patterns due to the T or R antigens. The results are summarized (Table 3) in three categories: the types that produce easily demonstrable M antigens, the types in which the M antigen has been difficult to demonstrate, and finally those types that can at present be identified only by agglutination pattern.

Table 1. *Serum opacity reaction of 508 strains of Streptococcus pyogenes typed by M precipitin tests*

The top half of the table records the reactions obtained with strains for which good anti-M precipitin sera were available; the lower half includes strains for which M precipitin sera are difficult to prepare.

Serological type	Stock strains		Routine strains		Total	
	Positive	Negative	Positive	Negative	Positive	Negative
1	0	18	0	37	0	55
2	10	1	19	0	29	1
3	0	25	0	12	0	37
5	1	18	2	10	3	28
6	0	14	0	9	0	23
9	14	0	10	0	24	0
10	0	4	—	—	0	4
12	2	14	6	37	8	51
14	0	8	—	—	0	8
15	1	9	—	—	1	9
17	0	13	—	—	0	13
18	0	8	0	50	0	58
19	0	8	0	8	0	16
23	1	0	—	—	1	0
24	1	10	—	—	1	10
26	0	6	1	0	1	6
29	1	7	—	—	1	7
30	0	4	—	—	0	4
31	0	4	—	—	0	4
33	0	6	—	—	0	6
36	0	7	—	—	0	7
37	0	1	—	—	0	1
39	0	4	—	—	0	4
43	1	10	—	—	1	10
	32	199	38	163	70	362
8	10	0	—	—	10	0
11	11	2	—	—	11	2
13	10	0	—	—	10	0
25	10	0	—	—	10	0
27	2	7	—	—	2	7
32	0	3	—	—	0	3
34	0	3	—	—	0	3
35	1	2	—	—	1	2
38	0	4	—	—	0	4
40	0	3	—	—	0	3
41	0	5	—	—	0	5
42	1	2	—	—	1	2
	45	31			45	31

— = not tested.

It has long been known (Todd & Lancefield, 1928) that the colonial differences apparent in many cultures of group A streptococci are sometimes related to the presence or absence of the M antigen. It was of interest to determine whether, when these variant colonies were picked and grown separately, the negative association of serum opalescence and M antigen would still be found. Two strains were studied in this manner. One, on isolation, had the typing 5/27/44 but gave no precipitin reaction with Type 5 antiserum. It was serum-opalescence positive.

Table 2. *Serum opacity reaction of 224 strains of Streptococcus pyogenes typed by T agglutination pattern*

Serological type	Stock strains		Routine strains		Total	
	Positive	Negative	Positive	Negative	Positive	Negative
4	24	1	47	2	71	3
28	11	1	37	3	48	4
1	—	—	0	2	0	2
6	—	—	0	1	0	1
9	—	—	0	1	0	1
14	—	—	2	1	2	1
22	—	—	27	0	27	0
8/25	—	—	17	5	17	5
5/11/27/44*	—	—	24	1	24	1
8/Imp.19	—	—	11	0	11	0
Imp.19	—	—	2	0	2	0
3/13/B3264	—	—	4	0	4	0
Total	35	2	171	16	206	18

* Recent work in our laboratory has shown that many strains of this typing pattern are in fact Type 11, for which an anti-M precipitin serum is now available, and some of the others have the agglutination pattern 5/27/44, which is characteristic of strains having the Type 5 M antigen. — = not tested.

A variant obtained from a single colony on subculture gave a Type 5 M precipitin reaction and did not produce an opalescence in serum broth. Similarly, a strain with the agglutination reaction 12 but not carrying Type 12 M antigen gave rise to a variant which did carry this antigen. The culture with M antigen did not give the serum reaction while the culture with no demonstrable M antigen produced the opalescence. That cultures sometimes consist of such mixtures giving serum opacity reactions around single colonies on serum agar plates had been noticed in the past (W. R. Maxted, personal communication).

Table 3. *Relationship of serum opacity reaction to serological type in Streptococcus pyogenes*

	No. tested	Serum opalescence reaction		Percentage positive
		Positive	Negative	
Good M antigen types*	439	72	367	16.4
Types from which M antigen can be extracted with difficulty or not at all†	229	191	38	83.4
Types recognized only by T agglutination pattern‡	64	58	6	90.6

* Types 1, 2, 3, 5, 6, 9, 10, 12, 14, 15, 17, 18, 19, 23, 24, 26, 29, 30, 31, 33, 36, 37, 39.

† Types 4, 8, 11, 13, 22, 25, 27, 28, 32, 34, 35, 38, 40, 41, 42.

‡ Typing patterns 8/25; 5/11/27/44; 8/Imp.19 and Imp.19; 3/13/B3264.

No association could be demonstrated between the serum opacity reaction and the production by the organisms of hyaluronidase or proteinase. Hyaluronidase preparations or crystalline proteinase had themselves no visible action on horse serum during $\frac{1}{2}$ hr. incubation at 37°. Extracts of streptococci, obtained from cultures known to produce a serum opacity reaction, by the use of urea (Krumwiede, 1954),

produced a pronounced opalescence when mixed with horse serum for $\frac{1}{2}$ hr. at 37° . Similarly, extracts prepared by lysis of washed streptococci with the phage-associated enzyme (Maxted, 1957) also produced opacity in horse serum even though they frequently did not possess proteinase or hyaluronidase activity.

The association of the serum opacity reaction with the lack of demonstrable M antigen did not appear to be due to alteration of the antigen since active extracts did not destroy isolated M antigen nor did the M antigen disappear from the surface of organisms when they were suspended in such extracts.

DISCUSSION

The results show that those strains of *Streptococcus pyogenes* which carry M antigen rarely produce the serum opacity reaction with the exceptions of Types 2 and 9. In contrast, serotypes usually producing the serum opacity reaction are those which elaborate M antigen poorly or not at all and are identified by their other surface antigens, either T or R. This relationship of the serum opacity reaction to the possession of M antigen is readily apparent from the results presented in Table 3. The isolation of M positive and M negative variants from both a Type 5 and a Type 12 culture and their resultant serum opacity reactions further demonstrated this relation. No association could be demonstrated between the serum reaction, streptococcal proteinase or hyaluronidase. These enzymes may reasonably have been expected to have an effect on the loss of M antigen from the surface had they occurred along with the lipoproteinase. Similarly, the lipoproteinase had no detectable activity on M antigen suggesting that the correlation is unlikely to be due to a direct interaction between this antigen and the factors responsible for the serum opacity reaction.

Krumwiede (1954) suggested that the serum opacity reaction was not due solely to a lipoproteinase but was more likely based on the interaction of a number of enzyme systems and accessory factors. She found no direct correlation between the degree of opalescence and the splitting of the lipoprotein. When, however, we tested active extracts obtained from four strains producing serum opalescence for the presence of factors capable of splitting horse serum lipoprotein, with each one such a splitting was found. Thus our studies may be interpreted as showing an inverse relationship between the presence of M antigen and the production of a specific enzyme.

Previous reports have also suggested an inverse relationship between the production of M antigen and particular enzymes. Williams (1954) noted that less than 1 % of 324 M precipitin positive strains but approximately 17 % of 371 strains identified by T agglutination pattern (or R antigen in Type 28) produced glucuronidase. Similarly, Crowley (1954) observed that streptococcal amylase was frequently elaborated by strains of *Streptococcus pyogenes* of Types 2 and 4, while only occasionally was it a product of other serological types. She found that while only 20 % of 121 strains typed by M precipitin reaction were amylolytic 62 % of 70 strains typed by agglutination pattern produced the amylase. Similarly, strong hyaluronidase producing strains are found more frequently among serological types such as Types 4 and 22 in which it is frequently difficult to demonstrate the presence of the M precipitating antigen. The present results on lipoproteinase support the impression,

held by many investigators, that strains carrying serologically demonstrable M antigen have a more limited array of certain enzymic capacities than those strains against which, in our laboratory, it is difficult consistently to prepare satisfactory antisera to their type specific M antigen.

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Form and Internal Structure of Cellular Aggregations in Early *Escherichia coli* Microcultures

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SUMMARY

A critical examination of results obtained with several microculture techniques showed that considerable variations may occur in the formation of cell aggregations, localized in definite zones in the preparation. The cellular aggregation studied in the greatest detail was the clonal microcolony. Time-lapse photomicrography of developing *Escherichia coli* microcolonies revealed that the arrangement of organisms follows an orderly pattern in both the smooth and rough phases. The genealogical history of the microcolony showed that cells are grouped by genealogical origin. The microcolonial configuration in both smooth and rough phases, at room temperature and 37°, consisted of closely packed arrays of organisms. At these temperatures, palisading appeared to be the primary movement through which the structure of the microcolonies was established. The usual palisading movement was of the sliding type, although buckling palisades were occasionally found in rough-phase cultures. At 44°, the microcolonial configuration in both smooth and rough phases did not invariably evolve through palisading. In these aberrant cases, the organisms appeared simply to push each other about, so that the final appearance was that of a loosely packed collection of organisms presenting as an irregular reticulum. Nevertheless, genealogical distribution remained orderly.

INTRODUCTION

The degree of order in a surface colony of bacteria must be great to allow for its regular form. A number of investigators have studied this phenomenon (reviewed by Knaysi, 1951), and they have suggested several hypotheses about the mechanisms involved. Nevertheless, as pointed out in *Topley & Wilson's Principles* (1955), we still know little of the internal structure of bacterial colonies. Two methods have been mainly used for studying this problem, impression preparations and direct microscopy of growing bacteria. There are, however, serious limitations in both these modes of investigation. Impression preparations do not allow tracing the development of a given microcolony from its inception in a single bacterium. The only record obtained from a constantly changing system is the stage at which cultivation is terminated. The series of developmental changes which occur, therefore, must be inferred from impression preparations of different microcolonies made at different time intervals. Another serious difficulty in this method arises from the fact that the preparations are fixed, rather than in the living state, when studied. Bouin's

fluid, the fixative used by Bisset (1938) in a study of impression preparations of bacterial colonies, has been found to cause considerable shrinkage of bacteria (Minck, Ebel & Minck, 1950). Thus the close approximation of bacteria which may be present in the living microculture would not be seen in fixed preparations. In the case of direct observation, the number of events under observation may be so large that it is impossible to become aware of some of them before the field has changed. It is for this reason that cell descent, for example, cannot be traced beyond the first few divisions.

The limitations of both these methods can be circumvented by time-lapse photomicrography of microcultures developing from an isolated organism. Although time-lapse photomicrography has been used for the study of the growth rates of individual cells (Bayne-Jones & Adolph, 1932), and for the study of the effect of antibiotics on growing cultures of *Escherichia coli* (Pulvertaft, 1952), it apparently has not been applied for a detailed analysis of microcolony formation. We, therefore, have re-examined this problem with time-lapse photomicrography of smooth and rough phase *Escherichia coli* in coverslip cultures. A genealogical notation was devised to aid in the frame-by-frame analysis of the developing microcolony. The observations obtained have illuminated a number of conflicting reports, and have also revealed some new aspects of the order present in clonal microcolonies. In the course of the study, it was also found that the clonal microcolony was only one of a number of aggregations which characteristically developed in microcultures. These other formations are briefly described.

METHODS

Establishing the microculture. The strain of *Escherichia coli* employed (ATCC no. 8677) was studied in both the smooth and rough phases. The rough phase was obtained by holding a smooth culture in brain heart infusion broth at 37° for 6 days. Isolation was from nutrient agar plates since the rough phase on beef brain heart agar was difficult to distinguish from smooth. The rough phase was verified by macrocolonial appearance on nutrient agar plates, the character of growth in nutrient broth, and by agglutination in salt solutions (Schneweis, 1959).

Both phases were grown in beef brain heart infusion broth for about 18 hr., and a heavily inoculated subculture was prepared and incubated for 1 hr. at 37°. Fifteen drops of the subculture were added with a capillary pipette to 5 ml. of diluted beef brain heart broth (4 parts water to 1 part medium). The diluted broth culture was then mixed with the pipette, which was used for inoculating the microcultures. A small number of cultivations were also made directly from both smooth- and rough-phase broth cultures which had been allowed to age for a week or more.

The principal microculture method used consisted of surface inoculation of a small thin film of agar on a coverslip. A no. 1 coverslip (22 × 30 mm.) was immersed in 95% (v/v) ethanol in water, drained by blotting one edge on a paper towel, and sterilized by flame. With the coverslip held at approximately 45 degrees to the horizontal, a drop of hot melted 2% water agar was allowed to run down the surface, leaving a thin streak of agar in the centre. After drying for 1 min., a drop of inoculum was placed on the agar streak and immediately drained off. The coverslip was left vertically in a Columbia jar (A. H. Thomas Co., Philadelphia, U.S.A.) for 2–5 min. It was then removed, and both ends of the agar streak were scraped off with a razor

blade to leave an area of about 3 mm.² in the centre. The agar film now had two cut edges and two edges formed from the free flow of agar (Fig. 1).

Two techniques were used for mounting the coverslip culture upon a glass slide. The method used for most of our observations is referred to as the 'open chamber' technique. In this method, the inoculated side was turned down, and the coverslip was then placed on two supports of no. 1 coverslips, approximately 1 cm. apart, which had been placed upon a 25 × 75 mm. glass slide. The four edges of the inoculated coverslip were sealed with melted paraffin to prevent drying. It was verified by direct observation that the inoculated surface of the agar film did not touch the glass slide after the coverslip had been mounted upon its supports.

The second method for mounting the culture, which we refer to as the 'closed chamber' method, consisted of dropping the inverted coverslip culture directly upon the glass slide and sealing the edges with paraffin (Fig. 2). This is essentially the method used in most previous studies.

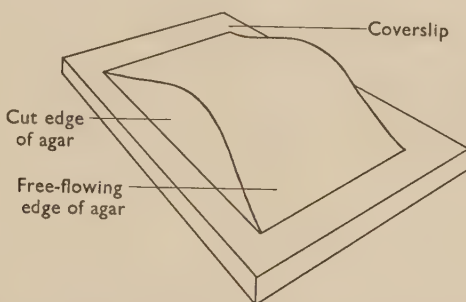


Fig. 1. Unmounted coverslip preparation for the open chamber method of cultivation.

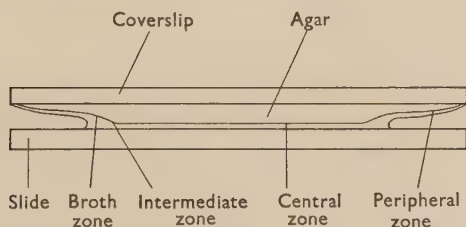


Fig. 2. Mounted coverslip preparation for the closed chamber method of cultivation.

Another method of microculture preparation tried was that of Fleming, Voureká, Kramer & Hughes (1950); this was used in only a few cultivations. Bacterial broth culture was smeared out on a coverslip, and immediately after the smear had dried, it was flooded with melted nutrient agar at 50°. The preparation was then mounted upon a glass slide. We refer to this procedure as the 'Fleming closed chamber' technique.

The completed preparations were mounted within a Zeiss microscope stage incubator. Incubation was carried out at room temperature (about 25°), 37° and 44°.

Photomicrograph methods. An isolated bacterium was located and observed with dark-phase contrast optics. In taking the photomicrographs, conventional light optics was used for the open chamber technique, and phase optics for the closed

chamber methods. The photographs were taken at 3 min. intervals with a Zeiss Contax camera, using a 100/1.30 oil-immersion objective lens. The condenser was not oiled. Kodak 35 mm. High Contrast Copy film (Micro-file) was chosen for its high contrast, high resolution and small grain size. The illumination, generally, was with Zeiss electronic flash, but a tungsten lamp was occasionally used. The best optical conditions for photography for both smooth- and rough-phase cultures were obtained with the closed culture methods, and particularly with the Fleming technique.

The photographic record was initiated as soon as possible after the bacterium was located, but the length of observation for the time-lapse sequence varied. In some runs it was greater than 4 hr., at which time members of the 7th generation were beginning to appear. In some cases incubation was carried out overnight, without time-lapse recording, to determine the effects of prolonged cultivation.

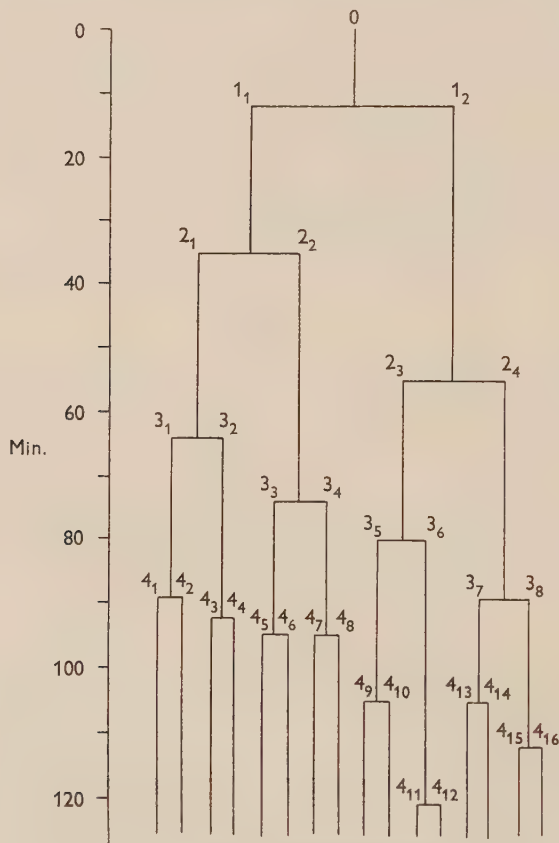


Fig. 3. Genealogical history of the clonal microcolony in Pl. 2, figs. 11-17.

Genealogy. Genealogical charts were drawn up to show descent in relation to time (Fig. 3). The original organism of the microcolony was designated as the 0th generation, and each succeeding generation was named in numerical order. The bacteria within a generation were given numerical subscripts. To distinguish between the two members of a pair arising from a division, that bacterium in the pair which

went on to divide first was numbered first. Thus, of the two bacteria of the single pair of the 1st generation, the first bacterium to divide was numbered 1₁, while the other member of the pair became 1₂. However, when the two sisters of a pair divided at the same time, the first member was chosen as that bacterium which gave rise to the first daughter to divide. In the case of the bacteria present at the end of the time-lapse sequence, the division pattern of course could not be known, and therefore differentiation between the members of a sister pair was not possible. These last bacterial pairs were named with subscript numerals nevertheless. Designations of organisms were entered directly upon the photographic print in the analysis of runs (Pl. 2, figs. 11–14).

RESULTS

Examination of the microculture preparations immediately after inoculation showed that the seeding consisted of well isolated single bacteria and occasional groups of 2 or even 3 which lay clumped in a parallel array (palisade). Following incubation, regardless of the method of cultivation, large variations in the developed cellular aggregates of both smooth and rough phases were found. These variations were limited to definite zones which were parallel to the two edges of freely flowing agar, and extended the full length of the agar from cut edge to cut edge (Fig. 2).

Peripheral zone

At the freely formed borders of the agar film in both the open and closed chamber methods, small cellular aggregations appeared early in cultivation. At 37°, the bacteria in this zone were highly motile in the initial stages of incubation, so that any aggregations of bacteria, with the exception of rough phase chains, were soon disrupted. At room temperature, these bacteria were only slightly motile. Smooth-phase daughter pairs tended to separate easily, while rough-phase organisms were found in motile chains of 4–8 members. The tenacity with which rough-phase bacteria remained attached to each other is illustrated in Pl. 1, fig. 1, which shows one member of an 8-membered chain blurred from motion, while the remaining bacteria are sharply in focus and exhibit no motility. These rough-phase bacterial chains characteristically developed angular folds (Pl. 1, fig. 2).

Within 2–8 hr. after inoculation, motility appeared to decline, on both smooth and rough cultures, and bacterial aggregates reappeared. With rough-phase organisms, slowing down occurred early in cultivation (2–4 hr.). The aggregates which then formed consisted of rods with considerable spacing between them, and with little or no order (Pl. 1, fig. 3). Overnight cultures showed bacteria of short lengths, comparable to those in the smooth-phase preparations, but the early disorder was still present. Although smooth-phase aggregates formed late in cultivation, the arrangement consisted of coccobacilli in a regular lattice (Pl. 1, fig. 4) which was maintained with continued cultivation. Both rough- and smooth-phase bacteria showed clearly discernible bridges of amorphous material between them, particularly in early stages of cultivation (Pl. 2, fig. 28).

Broth zone

In the closed chamber method only, a zone developed, with both bacterial phases, just medial to the periphery in which the bacteria were freely suspended and grew in a diffuse pattern similar to that seen in hanging-drop preparations from broth. We refer to this as the 'broth zone' (Fig. 2).

Intermediate zone

This area was a transitional zone, usually quite narrow, which lay between the broth and central zones. Smooth-phase bacteria in this area usually formed short chains (Pl. 1, fig. 5); rough-phase bacteria formed somewhat longer chains. A single case was observed, with a rough-phase culture, in which the intermediate zone was much wider than usual, and the chains of bacteria were considerably longer than those normally found (Pl. 1, fig. 6). At the inner edge of the intermediate zone were 'transitional' microcolonies (Pl. 1, fig. 5). From the side of the transitional microcolony which bordered on the intermediate zone, motile bacteria sometimes broke away and migrated into the intermediate zone. Non-motile bacteria remained in the colony, but they tended to separate widely from each other. The opposite side of these colonies, toward the centre of the preparation, resembled the structure of the typical central zone microcolony.

Central zone

The 'central zone' is the innermost area of the closed chamber microculture, and its typical aggregation of organisms is the clonal microcolony, a formation found in all the types of preparations studied. With smooth-phase microcultures, the clonal microcolony at 37° or room temperature consisted of closely packed arrays of bacteria whose general configuration appeared to depend upon the age of the original inoculum. When the inoculum consisted of 1 hr. culture which had been seeded from 18 hr. culture, the microcolony was quite regular and of a form which we refer to as 'compact'. From the time-lapse sequences (Pl. 2, figs. 11-17), it was apparent that post-fission palisading had an important role in establishing the compact configuration of the microcolony.

Typically, the first division of the original bacterium of the colony (0th generation) gave rise to a 2- or 3-membered chain (1st or 2nd generation). Continued growth along the long axis of the chain finally led to horizontal displacement of the proximal ends of a bacterial pair, and the bacteria then began to grow past each other (Pl. 2, figs. 11, 12). Several cases were seen, with the Fleming closed chamber technique, in which displacement of the end of a bacterium in the 2-member colony was vertical rather than horizontal. Palisading occurred in this case also. Further development of the compact microcolony was a consequence of cell growth, division and palisading. New palisades appeared only at the periphery of the microcolony.

When the microculture inoculum was obtained directly from a broth culture which had been held several days in the refrigerator, the microcolony configuration differed markedly from the compact forms obtained with an inoculum of vigorous young organisms. We now found microcolonial forms which we refer to as 'longitudinal'. In these cases bacteria of normal length (Pl. 2, fig. 24) or filaments exhibited reduced palisading, with the result that the bacteria accumulated as an

elongated configuration. The early development of filaments at a lateral edge of a colony, in particular, most profoundly affected the colonial configuration, since the possibility for early palisading at that edge was eliminated.

In some cases, the longitudinal microcolony soon began to develop an accumulation of bacteria at one or both ends, and this form was then referred to as 'longitudinal nodal'. In all cases, unless a persistent filament was present, all microcolonies which deviated from the compact form eventually filled out the irregular indentations in their outline. They then closely approximated the general outline of the compact form. Thus, following overnight incubation, it was found that the general outline of smooth-phase microcolonies was round, regardless of what the early configuration had been. Differences in diameter and degree of piling up of bacteria of the microcolonies in a single preparation were observed in all the methods of cultivation used.

Overnight cultivation at 37° or room temperature normally resulted in the formation of coccobacilli (Pl. 1, fig. 9), a finding similar to that observed in the peripheral zone of the microculture. Although our observations on this point were not thorough, it appeared that coccobacilli began to arise at about the 10th generation.

There was occasionally noted with rough-phase culture, in the closed chamber method, a persistent filament (Pl. 1, fig. 10) at the periphery of a microcolony, similar to the elongated L form variant described by Hughes (1953) in anaerobic microcultures of *Escherichia coli*. In general, single bacteria in the rough phase appeared to be morphologically indistinguishable from single bacteria in the smooth phase.

No evidence was found for segregation of bacteria by age. Only one case was noted, out of several dozen microcolonies recorded, in which one of the two bacteria from the first division failed to grow. With this single exception, all bacteria which appeared in the course of all observed microcolonies grew continuously, and in most cases divided when the original length of the organism had approximately doubled.

With dark phase contrast optics, a distinctive difference was observed between the peripheral and interior bacteria of a microcolony. The peripheral bacteria appeared black while the interior bacteria were much more transparent, showing internal granules, and almost suggesting a partial lysis (Pl. 1, fig. 7). These differences were not affected by the most careful focusing down through the depth of the clone. The bleached-out appearance of the interior bacteria, in fact, was found as soon as the organisms began to palisade, and always appeared to be associated with bridging (Pl. 2, fig. 29).

In rough-phase cultures at 37° and room temperature, the aggregations of bacteria were characterized by an angular zig-zag perimeter. Two main configurations were found. One consisted of a compact collection of bacteria, which we refer to as 'rough compact' (Pl. 1, fig. 7; Pl. 2, figs. 18-23). This colonial form appears to be the rough-phase equivalent of the compact colony of smooth phase cultures. The time-lapse series of a 'rough compact' colony (Pl. 2, figs. 18-23), however, emphasizes the distinctive characteristics which it has. These consist of angle formation between the bacteria, a rate of palisading much slower than smooth phase, and an irregular colony perimeter due to angular outcroppings of organisms. These outcroppings eventually give rise to columns of bacterial chains. The aggrega-

tion now begins to transform into a quite different aspect which may be described as 'rough nodal' (Pl. 1, fig. 8). This consists of a column of bacteria along which are interposed compact nodes. The rough nodal configuration was also observed to develop directly from chains of bacteria without the initial formation of the rough compact grouping. Both of the rough-phase microcolonial forms were observed with all the methods of cultivation. Eventually, the continued production of organisms rounded out the irregular outline of the rough nodal form so that it resembled the compact configuration.

The primary post-fission movement in rough phase at 37° and room temperature also was palisading. As a general rule, palisading was through the same process as in smooth-phase microcolonies. The rate at which palisades developed, however, was much slower than in smooth-phase cultures. The difference in palisading rate can be readily seen by comparing smooth- and rough-phase microcolonies at approximately the same bacterial counts. We have done this at the 12- to 15-organism stage in Pl. 2, figs. 15, 22 and 25. Both the smooth-phase compact colonies shown (Pl. 2, figs. 15, 25) have all their organisms involved in at least one palisade, while the rough-phase colony (Pl. 2, fig. 22) has only half its organisms palisaded.

Two instances were observed early in cultivation of rough phase bacteria at 37° in which palisades were created by the folding or buckling of a chain of bacteria (Pl. 3, figs. 37, 38). Both these cases developed in the Fleming closed chamber.

On tracing the family history of the bacteria in a microcolony, from either smooth- or rough-phase culture, it was found that in most cases the descendants of organism I_1 could be separated from the descendants of organism I_2 by a long axis running through the centre of the microcolony (Pl. 2, fig. 17). In some cases this regularity was disturbed by an aberrant bacterium. Thus, disruption in one microcolony was found to be due to a single bacterium which had deviated medially during the formation of an early palisade (Pl. 2, figs. 24-27), and which had consequently penetrated into the mass of bacteria produced in the other line. Sublines of organisms I_1 and I_2 were also usually found to be separated along axes parallel to that separating the I_1 and I_2 lines.

The results obtained from the cultivations at 44° were strikingly different in some respects from those obtained at 37° and at room temperature, with both smooth and rough phases. There was a greater tendency for filaments to appear, but the most striking difference was the absence of sliding palisades (Pl. 3, figs. 30-36). In these cases, the configuration was established simply by the bacteria pushing each other about. This led, in some instances, to buckling palisades. The extracellular material which formed broad bridges between bacteria when palisading occurred at 37° was found confined to narrow strands which did not shift in position as the bacteria were pushed about. Few of these strands were present. When bacterial lines were identified, it was clear that genealogical distributions were as orderly as at 37° (Pl. 3, fig. 36).

DISCUSSION

The hypothesis of Dean & Hinshelwood (1957) that the irregularities of the agar surface play an important role in the order of organisms in microcolonies had led us to a close examination of the entire surface of our preparations. However, the large differences in the ordering of organisms which were consequently detected in the different zones of the open and closed chamber preparations appear to be due largely

to variations in the thickness of the surface film of fluid, one extreme of which led to the 'broth' zone, detected in the closed chamber preparations only, and the other extreme of which led to early piling up of bacteria in the microcolony. As illustrated in Fig. 2, the broth zone in all probability is in the meniscus formed by the border of the broth film on the agar coming into contact with the glass slide.

Our observation that motile *Escherichia coli* organisms broke away only from the side of late 'transitional' microcolonies bordering on the intermediate zone also may be explained by film thickness. Apparently the intermediate zone film of fluid tapers off from the maximum thickness of the broth zone to the minimum film thickness of the central zone.

It is possible that the observation of Pearce & Powell (1951) that motile forms begin to appear in microcolonies of *Bacillus subtilis* after incubation for 4-5 hr. was made on transitional microcolonial forms. These investigators suggested that motility develops because of an increase in the fluid film in which the organisms lie. Presumably the metabolic products of the bacteria alter the osmotic balance in the surface film sufficiently to extract water from the underlying agar. This hypothesis does not appear to be pertinent to our system in view of the characteristic location of motile organisms in the transitional microcolonies only on the side toward the periphery of the preparation.

The intercellular bridging in the bacterial aggregations of the peripheral and central zones is open to at least two interpretations. Some of the bridges suggest an exchange of material between the organisms. This appearance strongly resembles published photographs of presumptive bacterial conjugation (Lederberg, 1956). From our own observations, however, the 'conjugation' configurations of Lederberg may be nothing more than the early stages of palisading (compare fig. 1 of Lederberg, 1956, with Pl. 2, fig. 19). It appears more probable to us that the bridging observed in the present study was simply due to adhesion through capsular material. The difference in optical density between peripheral and interior bacteria of the microcolony with dark-phase optics may be an artefact.

It is apparent that characteristic microcolony formations of the central zone appeared quite early, and could be recognized in most cases almost as soon as the minimal number of bacteria required for a formation had appeared. The early microcolonies presented a graded series of forms, which were repeatedly demonstrated in preparation after preparation. Our observations of both smooth and rough phases of *Escherichia coli* are essentially in agreement with those of Nutt (1927) made by direct microscopic study of *Salmonella aertrycke*, *S. paratyphosa* B and *S. enteritidis*, and with those of Seal (1937) who studied cholera vibrios by the same direct method as did Nutt.

On the basis of morphology alone, no cellular difference was discernible between smooth- and rough-phase organisms of *Escherichia coli*, a point which Hu (1936) had earlier emphasized in microculture studies of *Salmonella typhimurium*. We did not observe any tendency in rough phase for the growth of the extremely long filaments of the medusa-head colony, which Bisset (1938) considered to be the typical expression of the rough phase in *Escherichia coli*. Our rough-phase microcultures, rather, resembled closely those described by Nutt (1927) and Seal (1937). Schneweis (1959), similarly, found that only 2 of 7 strains of rough-phase Enterobacteriaceae gave medusa-head colonies in microculture.

Palisading, the primary movement by which our bacteria aggregated, cannot be considered a cultural artefact resulting from mechanical restraint, as suggested by Bisset (1950), since we found it in both the closed and open chamber techniques at 37° and room temperature. Graham-Smith (1910) investigated this possibility 50 years ago by following the development of colonies on the surface of agar in Petri plates; he concluded that mechanical restraint did not account for palisading. Among the new evidence obtained in the present study bearing on this problem is the observation, with the Fleming closed chamber method, of a vertical displacement of a proximal cell end, and the subsequent appearance of the palisade, with the bacteria continuing to lie in two planes. It would seem that if any pressure from the agar were present it would have forced the bacteria back into the same horizontal plane. Clonal microcolony formation without palisading, in agreement with Bisset's point of view, was observed only in cultivations at 44°. It is possible that the capsular material, through which the palisading movement appears to occur, undergoes a transformation from a sol to gel at the elevated temperature.

The regular distribution by bacterial lines of the bacteria within the clonal microcolony demonstrates the high degree of order present. Previous failures to appreciate this (Bisset, 1938; Schneweis, 1959) undoubtedly were due to a failure to follow the genealogical history of the microcolony.

The dynamics of microcolony formation at 37° appear to depend primarily upon the fact that growth is in the direction of the longitudinal axis of the bacterium. The naturally undisturbed order, therefore, would be that of a chain of bacteria. As the microcolony begins to exhibit palisading, the concept of available free space, developed on the basis of fibroblast tissue culture studies (Abercrombie & Heaysman, 1954), becomes illuminating. The bacterial microcolony, once palisading is initiated, may be regarded as a spatial distortion of a single chain of bacteria. When the palisading movements have established 3 or more columns of bacteria, the exterior organisms will continue to form new palisades, but the interior columns will develop unbroken because of constraint by the adjoining organisms. The interior columns, therefore, have reverted to the initial form of aggregation, with the new possibility of growing into considerably longer chains. From preliminary studies of overnight cultivations (not described in the present report), it is apparent that extension is not unlimited. Centrally located interior bacteria are forced up above the plane of the original monolayer, and a second smaller layer of bacteria develops. As the process continues, the typical convexity of a large colony gradually evolves. The interplay of movements and constraints regularly leads to a circular colony form regardless of the early microcultural configuration. At 44° with the loss of palisading as a factor in the aggregation of the bacteria, close packing does not occur, but the formations nevertheless eventually become circular also. The bacterial microcolonial forms we have described resemble, in some respects, the monolayer aggregations of fibroblasts in tissue culture. Abercrombie & Heaysman (1954) found that the adhesion of fibroblasts to each other results in a characteristic meshwork which has a striking resemblance to the open aspect of our *Escherichia coli* microcolonies at 44°. The direction of movement of fibroblasts, once they have contacted each other and are adhering, is controlled by the position of the free space. Consequently, whatever the original shape of the culture, it will tend to assume a circular form as it continues to grow. A considerable number of problems

remain to be clarified before the mechanisms of bacterial colony formation will be understood. With elucidation of this phenomenon, it may well be that new light may be thrown upon the more complicated processes of cell aggregation in higher phyla.

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EXPLANATION OF PLATES

PLATE 1

Figs. 1-4. Cellular aggregates in peripheral zone of the microculture. Dark-phase contrast, $\times 1250$.

Fig. 1. Motile chain of rough-phase microculture (arrow indicates motile organism).

Fig. 2. Angular folding in bacterial chain; rough-phase microculture.

Fig. 3. Aggregation of rough-phase organisms after 5 hr. incubation.

Fig. 4. Aggregation of smooth-phase organisms following overnight incubation.

Fig. 5. Transitional microcolony in smooth-phase culture, overnight incubation; closed chamber method of cultivation. Dark-phase contrast, $\times 1250$.

Fig. 6. Rough-phase organism chains in the intermediate zone; overnight incubation. Dark-phase contrast, $\times 1250$.

Figs. 7-10. Cellular aggregations in central zone. Dark phase contrast, $\times 1250$.

Fig. 7. Compact rough clonal microcolony after 4 hr.; Fleming method of incubation.

Fig. 8. Nodal rough clonal microcolony following overnight incubation; open chamber method of cultivation.

Fig. 9. Coccobacilli in smooth-phase microcolony; overnight incubation; open chamber method of cultivation.

Fig. 10. Peripheral filament in rough-phase microcolony following overnight incubation; closed chamber preparation.

PLATE 2

Figs. 11-17. Time-lapse series of compact smooth-phase clonal microcolony; times are given at top of each figure, with 0 time taken at first division. Bright field optics; open chamber preparation; $\times 1800$.

Figs. 11-14. Genealogical identification of the bacteria in the clone.

Fig. 17. Spatial distribution of bacterial lines 1_1 and 1_2 in a well developed clonal microcolony.

Figs. 18-23. Time-lapse series of a compact rough clonal microcolony; Fleming method of cultivation. Dark-phase contrast optics; $\times 1800$.

Figs. 24-27. Time-lapse series of longitudinal compact clone, with disrupted spatial distribution of cell lines, 1_1 and 1_2 ; open chamber method. Bright field optics; $\times 2000$.

Fig. 28. Intercellular bridging of coccobacilli in the peripheral zone of the culture. Dark-phase contrast, $\times 4800$.

Fig. 29. Intercellular bridging of rough-phase organisms in a clonal microcolony of the central zone. Dark-phase contrast; $\times 4800$.

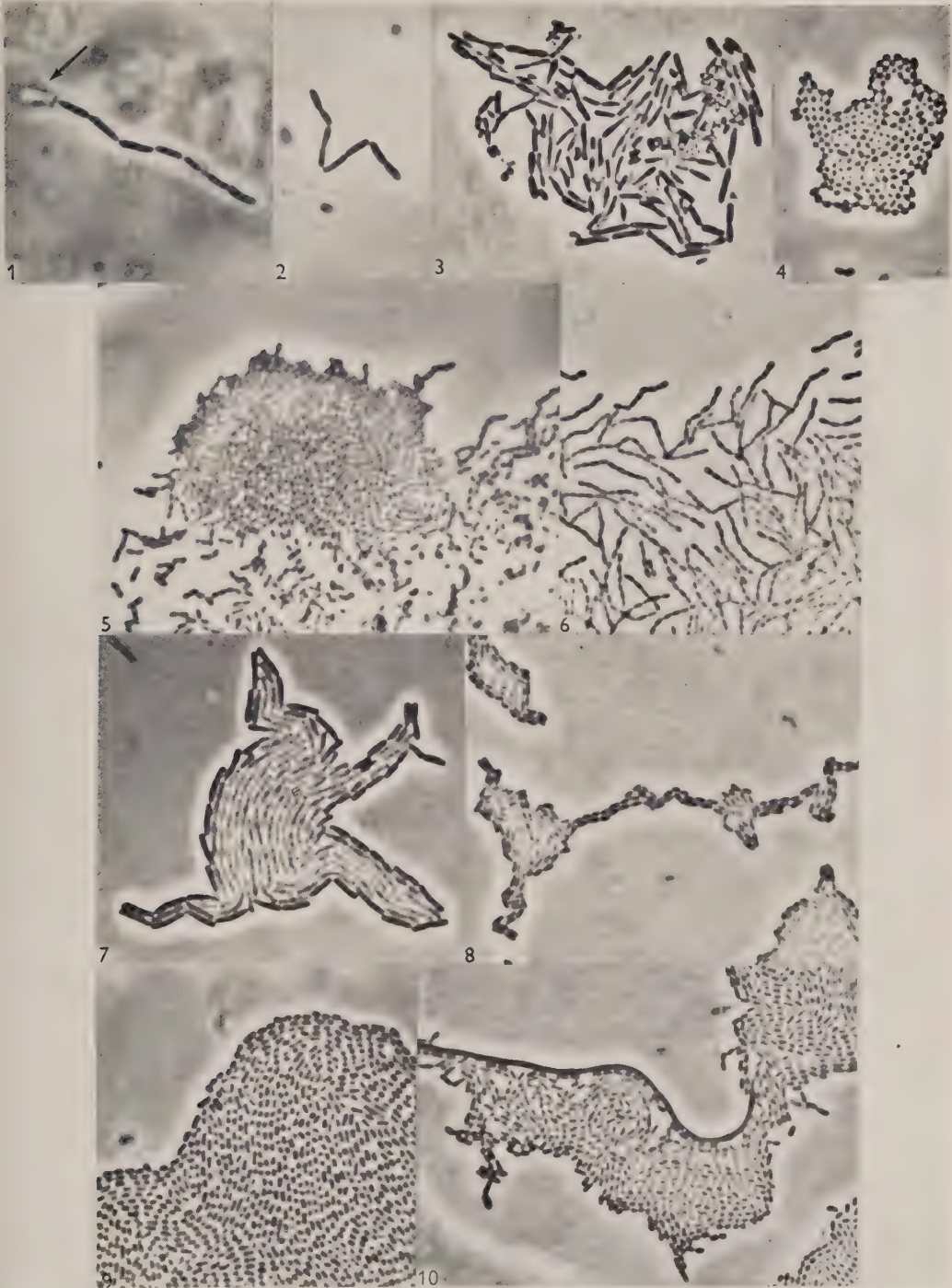
PLATE 3

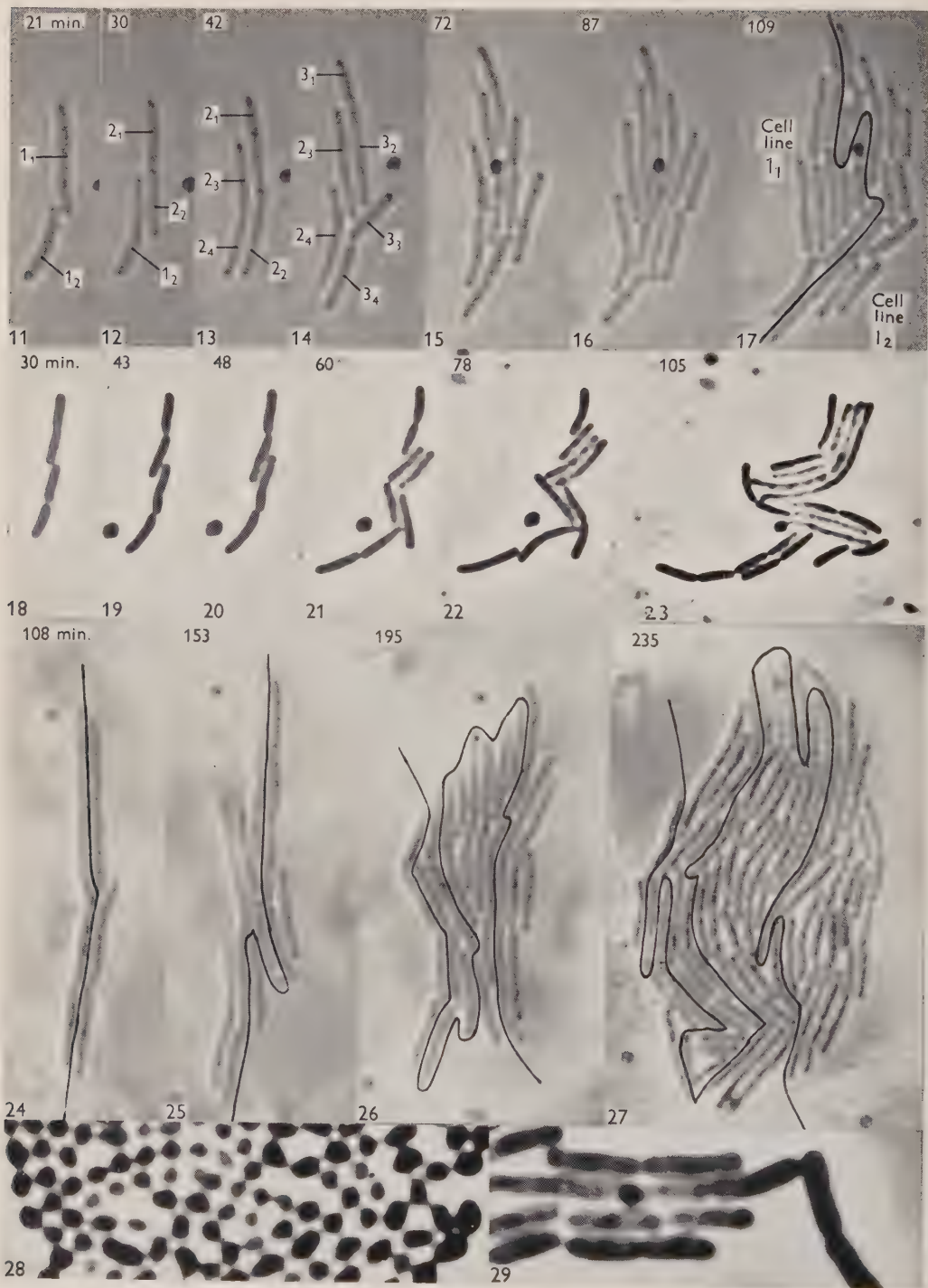
Figs. 30-36. Time-lapse series of smooth-phase microcolony (young inoculum) at 44° . Dark-phase contrast optics, Fleming technique; $\times 1250$.

Figs. 30-35. Early stages in the development of the microcolony; note the parallelism attained simply through pushing about of bacteria. Capsular adhesions are almost entirely absent (*a* indicates an abortive sliding palisade; *b* illustrates parallelism of organisms through a buckling movement).

Fig. 36. Configuration of the well developed clone, with genealogical distribution of bacterial lines 1_1 and 1_2 . Note the open appearance of the colony.

Figs. 37, 38. Initial movements in the formation of a buckling palisade at 37° in the rough phase; Fleming method of cultivation. Dark-phase contrast optics; $\times 1800$.







The Effect of Light on the Developmental Cycle of *Nostoc muscorum*, a Filamentous Blue-Green Alga

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SUMMARY

The Allison strain of *Nostoc muscorum*, when cultivated in complete darkness, differs morphologically from the forms which prevail in the light. When cultivated in the dark with glucose as energy and carbon source, the alga grows very slowly as a mass of large undifferentiated cells (the aseriate stage). Exposure to small amounts of light or the addition of aqueous extracts of light-grown cells enables development of typical nostocacean filaments. The aseriate morphology is a transient stage of development found in all autotrophic or photoheterotrophic cultures. By growing *Nostoc* in complete darkness and then exposing to light of low intensity, synchronous development occurs. The cyclic sequence of development is described. The inhibition of growth in the dark is interpreted as a lack of morphogenetic substances formed only in the light.

INTRODUCTION

Early investigators of the Hormogonales observed that many of these filamentous blue-green algae, collected from natural sources, displayed differentiated cells of two types: akinetes (gonidia) and heterocysts (De Bary, 1863; Janczewski, 1874; Bornet & Thuret, 1880; Brand, 1903). Certain filaments or portions of filaments (trichomes) exhibited specialized structure or function. These filaments, if motile, were termed hormogonia, if non-motile, hormospores (hormocysts) (Thuret, 1844; Borzi, 1879; Bornet & Flahault, 1886; Borzi, 1916). In addition, many of these organisms were found in nature to occur as macro colonies of characteristic morphology. The filamentous elements of each colony were, at times, arranged in a regular fashion (Bornet & Thoret, 1880; Geitler, 1925; Fremy, 1930).

Evidently, a description of the life history of the filamentous blue-green algae must account for the formation of specialized cells and filaments. Nevertheless, a sequence of events explaining the formation and function of the specialized elements of the cyanophycean thallus has never been established. Instead, there arose the concept that development of the filamentous blue-green algae was a simple vegetative process analogous to reproduction of the true bacteria. This notion has been supported by the apparent cytological similarity of cyanophycean and bacterial cells, as well as by the recognition that the filamentous bacteria classified as Beggiatoaceae may be colourless relatives of Hormogonales, notwithstanding the dubious relationship of the Beggiatoaceae to the true bacteria (Pringsheim, 1949).

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Although pure cultures of the filamentous blue-green algae have been available for at least 40 years, little attention has been paid to the developmental morphology of these organisms (Pringsheim, 1913, 1946). The most notable pure culture studies were performed by Harder (1917*a*), who clearly showed that the production and subsequent growth of hormogonia were a means of new colony formation. These observations were in substantial agreement with those of Thuret (1844), Sauvageau (1897) and Geitler (1921) who studied similar phenomena in nature or in crude cultures.

Although the formation and germination of akinetes have been described by many investigators (Fritsch, 1904, 1945), the function and development of the heterocyst has not been elucidated despite the keen interest of phycologists in this enigmatic structure (Fritsch, 1951). Except for the observations of Harder (1917*b*) and Fogg (1949), the effects of chemical or physical agents upon the development of the structures of the blue-green algal thallus have been neglected.

Allison, Hoover & Morris (1937) described a strain of *Nostoc muscorum* capable of growth in the dark with either glucose or sucrose. In the present investigation, such heterotrophic cultures were found to differ morphologically from cultures grown in the light. Dark-grown cultures resembled old light-grown cultures of this organism as reported by Allison *et al.* (1937) and the stage of hormogone 'germination' described by Thuret (1844) and Sauvageau (1897). However, the morphology characteristic of the dark-grown alga could always be found as a transient but recurrent phase in cultures actively growing in the light. These observations suggested, and our experiments confirmed, that in the light a sequence of developmental stages occurred which was not completed during the slow but sustained growth which prevailed in the dark. The slow growth of the algae in the dark may be related to their inability to complete a developmental cycle under these conditions.

METHODS

The organism used in these studies was a strain of *Nostoc muscorum* kindly supplied by Dr F. E. Allison several years ago (designated *Nostoc muscorum* A).

The following two basal media were used: Medium 1 (modification of the medium described by Fogg (1942) for the cultivation of *Anabaena cylindrica*): K_2HPO_4 , 0.15 g.; $MgSO_4 \cdot 7H_2O$, 0.20 g.; $CaCl_2 \cdot 2H_2O$, 0.025 g.; $FeCl_3 \cdot 6H_2O$, 2.0 mg.; $MnCl_2 \cdot 4H_2O$, 0.4 mg.; Na_2MoO_4 , 0.4 mg.; H_3BO_3 , 0.6 mg.; $CuSO_4 \cdot 5H_2O$, 0.04 mg.; $ZnSO_4 \cdot 7H_2O$, 0.04 mg.; distilled water to 1 l. Medium 2 (a chelated solution): K_2HPO_4 , 0.15 g.; $MgSO_4 \cdot 7H_2O$, 0.20 g.; $CaCl_2 \cdot 2H_2O$, 0.025 g.; disodium ethylenediamine tetraacetate, 0.05 g.; $FeSO_4 \cdot 7H_2O$, 8.0 mg.; $ZnCl_2$, 10.0 mg.; H_3BO_3 , 1.0 mg.; $MnSO_4 \cdot 4H_2O$, 8.0 mg.; $CuCl_2 \cdot 2H_2O$, 1.0 mg.; $CoCl_2 \cdot 6H_2O$, 1.0 mg.; Na_2SiO_3 , 0.025 g.; Na_2MoO_4 , 3.0 mg.; distilled water to one litre.

In both these media, the first three salts were made up, as individual stock solutions. The other constituents were combined in a single stock solution made up at $\times 100$ the concentration desired in the final medium, and adjusted to pH 4-5 with HCl. Both media were adjusted to pH 7.2 before autoclaving.

Substances added to the basal media were sterilized separately by autoclaving, or by filtration through a Seitz or sintered-glass filter, and were added aseptically to the culture vessels. Liquid cultures were maintained in Pyrex culture tubes or

50 ml. Erlenmeyer flasks, each containing 10 ml. basal medium. In experiments with solid media, 1.5 % (w/v) agar was added to the nutrient solution before autoclaving. Any separately sterilized materials were added, after sterilization, to the cooled but still liquid agar. After mixing, about 12 ml. molten medium were poured into sterile Petri dishes and inoculated by spreading suspensions of algal material on the surface with sterile bent glass rods. Inoculum suspensions were routinely prepared by homogenizing the harvested alga for 30 sec. in a sterile Waring Blendor cup with a small amount of sterile basal medium. The suspensions were washed by sedimentation in a centrifuge and resuspended in sterile basal medium two or three times. The developmental morphology of *Nostoc muscorum* A was examined in cultures grown in light or darkness, on solid or liquid media, at a temperature of $24 \pm 2^\circ$. Cultures were illuminated with fluorescent lights and screened with layers of cheesecloth until the desired intensity, as measured by a Weston light meter with daylight filter, was obtained. Dark cultures were incubated in specially constructed light-tight boxes.

Photomicrographs were taken with a 'box camera' on Kodak Panatomic X film, through a Spencer research microscope.

RESULTS

When *Nostoc muscorum* A was cultivated in the light on the surface of solid inorganic media, the following phases of development were observed (Pl. 1, figs. 1-8): motile trichome stage, aseriate stage, stage of rapid filament growth, dissolution of slimy envelope with consequent loosening of tightly packed filaments, and breaking of filaments at heterocysts to yield motile trichomes (hormogonia). The term aseriate stage is proposed for that phase of development in which the cells occur in packets and display no linear attachment to one another in continuous chains. During this study, it remained a moot point whether or not the cells within the packets were attached. Subsequently, it has been possible to show that the aseriate packets consist of groups of single cells or short chains, up to 3 or 4 cells in length, confined within a common sheath. The anastomoses of these cells to form continuous filaments will be described in a future paper. The hormogonia migrated freely over the surface of the agar in moving streams of filaments. They were often seen to aggregate in revolving spiral formations. Such filaments came to rest, and cell divisions took place in a plane parallel to the axis of the filament. Succeeding cell divisions produced clusters of cells (the aseriate stage) from each of the cells of the original hormogone, except that the terminal cells developed into terminal heterocysts. At the aseriate stage the large cells in each cluster did not immediately show a linear relationship to each other. However, upon subsequent development filamentation of the clumped cells occurred with the differentiation of intercalary heterocysts in the rapidly elongating new filaments. Breakage of filaments at the intercalary heterocysts liberated heterocysts and hormogonia.

The morphology characteristic of the organism grown for 2-4 weeks in a liquid inorganic medium at a light intensity of 200 ft.-candles is shown in Pl. 2, fig. 9. When the inorganic basal medium was supplemented with 1 % (w/v) sucrose or glucose very slow growth occurred in complete darkness. The morphology exhibited by the alga after growth for several weeks in the dark is shown in Pl. 2, figs. 10-12.

The relationship between light intensity and dry weight of alga produced was examined in the following manner. Cardboard boxes, with cellophan windows on their under sides, were fitted with light-tight lids. The boxes were painted on the outside with aluminium paint, and on the inside surfaces with 'flat black'. The boxes were placed on glass shelves illuminated from below by a bank of fluorescent lights. By interposing various thicknesses of cheesecloth between the glass shelf and the cellophan windows of the boxes, any desired light intensity could be obtained. Culture tubes containing 10 ml. of chelated medium 2, with or without 1 % (w/v) separately sterilized glucose were arranged in the boxes in such manner as to receive the light coming from below. Nine boxes of this type were set up for observing growth under light intensities ranging from 1.0 to 450 ft.-candles. Similar culture tubes were inoculated simultaneously and incubated in complete darkness. The inoculum consisted of a loopful of a homogenized suspension prepared from a culture grown with agitation for 2 weeks on inorganic medium at a light intensity of 250 ft.-candles. After incubation for 22 days, the experimental cultures were harvested by centrifugation. After washing the organism twice with distilled water, pooled replicates were transferred to tared weighing bottles, and dried to constant weight in a forced-draft oven at 67°.

Table 1. *Growth as a function of light intensity and presence of glucose*

Each tube contained 10 ml. of Medium 2. Cultures were incubated for 22 days at $24 \pm 2^\circ$. Dry weights were obtained by pooling the contents of four replicate tubes, washing the algal contents, and drying to constant weight in tared weighing bottles.

Light intensity (ft.-candles)	mg. dry weight of algal growth/tube	
	Medium 2	Medium 2 + 1 % glucose
0	No growth	Visible growth, not weighable
1.0	No growth	Visible growth, not weighable
4.5	No growth	1.2
22	Visible growth, not weighable	3.8
45	1.1	6.7
80	1.4	15.8
160	3.1	6.1
200	3.1	10.8
290	4.8	12.7
450	4.3	5.8

Dry weights of algal material grown in tube cultures after incubation in darkness and at various light intensities are given in Table 1. Light intensities which were insufficient for growth under photoautotrophic conditions produced a measurable growth response in tubes containing glucose. Growth was thus stimulated by glucose when photosynthesis was limited solely by low light intensity. Apparently light stimulated the use of glucose for growth. The quantity of growth which occurred in complete darkness was insufficient for dry weight measurement.

Figure 1 illustrates the development of cultures grown at different light intensities in media containing glucose. It may be noted that the morphological stages of development, described above, were correlated with growth which took place

between 0 and 80 ft.-candles light intensity. This is the range at which light intensity is nearly proportional to growth response. Above 80 ft.-candles, growth decreased sharply, then resumed as the light intensity increased still further. From 80 ft.-candles all tubes showed long filaments with intercalary heterocysts. Motile trichomes were not being formed and therefore the morphological stages associated with their further development were absent.

The response of *Nostoc muscorum* A to different light intensities suggests that cultures which have grown in complete darkness for extended periods might be inhibited in their development, either by the lack of essential substances formed only in the light, or by the accumulation in the dark of inhibitory material. When dark-grown cultures, exhibiting the aseriate morphology shown in Pl. 2, fig. 11, were

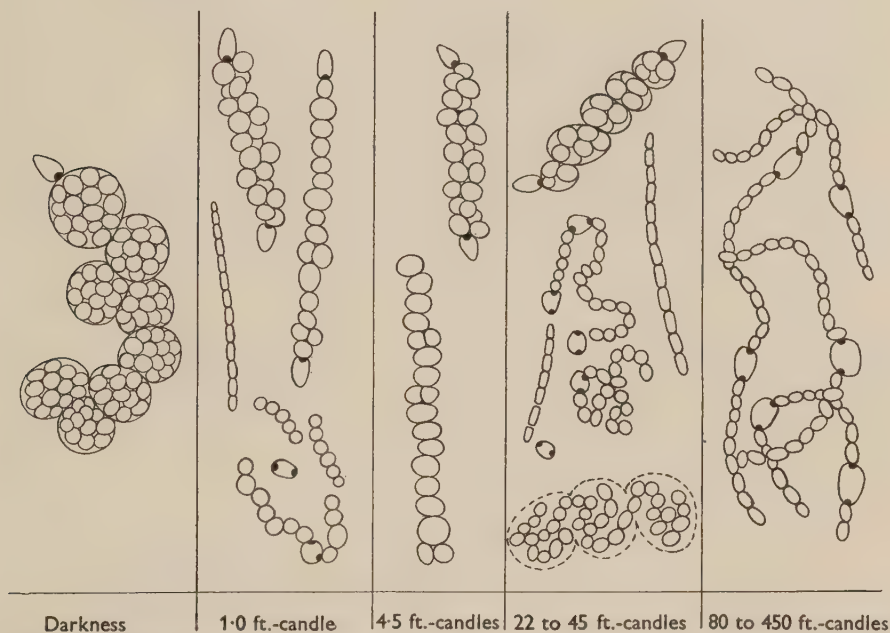


Fig. 1. Morphology of *Nostoc muscorum* A after 22 days in medium 2+1% glucose at different light intensities.

placed in light of 50 ft.-candles intensity, rapid growth took place with a synchronized development of filaments similar to growth observed on solid media. Plates 2 and 3 show the developmental stages found in synchronous cultures. Figure 2 presents an over-all scheme of the developmental cycle of *Nostoc muscorum* A deduced from those observations.

In accordance with the results obtained by Allison *et al.* (1937), only glucose, sucrose and fructose were found to support the growth of this organism in the dark. The following compounds and mixtures were ineffective as sole carbon sources in the dark: cellobiose, maltose, lactose, glycerol, pyruvate, succinate, citrate, lactate, acetate, urea, extract of *Nostoc muscorum* A, yeast extract, beef extract, malt extract, soil extract, plankton extract, casein hydrolysate, peptone, tryptone, nutrient broth, trypticase-soy broth, either alone or in combination or when sup-

plemented with NH_4Cl or KNO_3 . Growth in the dark on medium 2 + 1 % glucose or sucrose was not stimulated by additional glucose or sucrose, or, with one exception, by the substances listed above. An extract of light-grown *Nostoc muscorum* A, when added to medium 2 + 1 % glucose or sucrose, markedly enhanced growth in the dark. The extract was prepared from algae grown with agitation in medium 2 at 250 ft.-candles. About 50 g. wet weight of algal material was ground

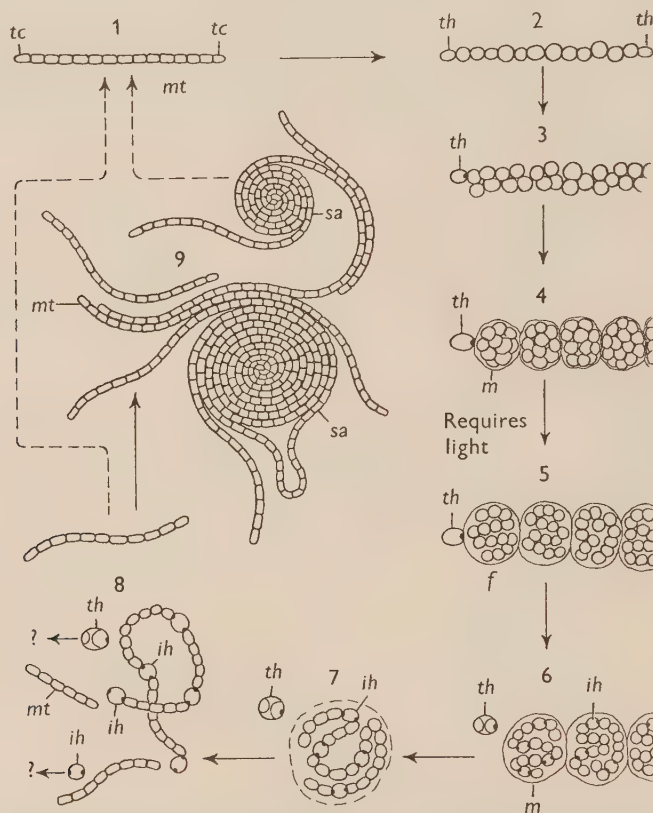


Fig. 2. A diagram of the developmental sequence indicated by arrows and numbered stages. Arrows with dotted shafts indicate that information concerning this portion of the cycle is incomplete. Stage 1 consists of the hormogone or motile trichome (*mt*) with tapered terminal cells (*tc*). In stage 2 the terminal cells begin differentiation to terminal heterocysts (*th*) while the intercalary cells elongate prior to division in the plane parallel to the axis of the trichome. In stages 3 and 4 division of the intercalary cells continues until a cluster of cells surrounded by a sheath or membrane (*m*) has formed from each of the intercalary cells of the original trichome. Stage 5 is characterized by the filamentation (*f*) of the cells within each ensheathed cluster. In stage 5 intercalary heterocysts (*ih*) are seen in the newly formed filaments. At stage 7 the membranes enclosing each filament cluster begin to break down. During stage 8 the filaments break at the intercalary heterocysts, liberating hormogonia and heterocysts. The hormogonia swarm and may cohere in moving spiral aggregates (*sa*) seen in stage 9. Such hormogonia are capable of repeating the cycle of development.

with sand in 250 ml. distilled water. After grinding, the sand was separated by centrifugation and the yellow supernatant fluid sterilized by filtration through a sintered-glass bacterial filter. The addition of as little as 0.2 ml. of this extract to

10 ml. medium significantly stimulated growth in complete darkness. Microscopical examination indicated that the presence of the extract had enabled the organism to pass through the various developmental phases previously observed only in the light: actively motile trichomes swarmed up the sides of the tubes as evidenced by streams of hormogonia in the abundant parenchymatous growth material clinging to the tube walls. It has not yet been possible to obtain a sufficient amount of dark grown algae (aseriate stage) to prepare a suitable control extract of dark grown cells. Such an extract should be prepared in complete darkness to have a true control. Large inocula of light grown *Nostoc* will not become aseriate in the dark, presumably due to carry-over of some factor required for filamentation.

Although growth in the light exhibited all developmental stages regardless of whether the organism was grown under autotrophic or heterotrophic conditions, the presence of glucose exerted additional effects which were not found with sucrose. Superficially this effect was the inhibition of motile trichome formation. In cultures developing in the presence of glucose in the light, the stage of rapid seriate growth and further differentiation of heterocysts was prolonged, yielding extremely long non-motile filaments (Pl. 3, fig. 19). These filaments eventually broke at an intercalary heterocyst, but breakage occurred between the heterocyst and only one of its adjacent cells. Consequently, filaments were formed with intercalary type heterocysts in terminal positions, instead of free heterocysts which arose in the absence of glucose. The filaments so produced were capable of oscillatory motion and produced the aseriate phase of development (Pl. 3, fig. 20).

The macroscopic appearance of cultures grown in the light in media containing glucose reflected the effect of glucose on trichome development. Whereas cultures grown in inorganic media or in media containing sucrose, or fructose, in the light, were tufted and crustose, cultures grown in media containing glucose, in the light, appeared as a loose non-adherent mass of long filaments.

DISCUSSION

The observation of a sequence of morphological stages in cultures of *Nostoc muscorum* A suggested that a regular developmental cycle exists for this organism. The interruption of development by darkness could not be explained by a requirement of photosynthesis for growth, since the organism can maintain a slow but definite growth in the complete absence of light. Therefore, it was postulated that a factor essential for the completion of the developmental cycle was formed only in the light. In its absence *N. muscorum* A assumes the aseriate morphology when grown in a medium containing glucose, or may produce randomly arranged short trichomes in the presence of sucrose. Bringing the cultures into light causes a resumption of development through the regular sequence of changes observed on solid inorganic media. The light-induced development of dark-grown cultures is synchronous, allowing a detailed study of morphological changes. Not only is the light intensity required to restore development well below the minimal amount needed for photo-autotrophic growth (see Table 1), but the developmental sequence is restored when dark grown cultures are exposed to low light intensities for as little as 30 min. and then returned to complete darkness. The existence of the hypothetical substance required for development was made more likely when it was

found that the addition of extracts prepared from *Nostoc* grown in the light permitted completion of the development of cultures in complete darkness, and substantially increased the rate of growth. As yet the rate of growth in darkness, in media containing extracts of light grown cells, has not equalled the rate of growth in the light.

The concept that filamentous members of the Cyanophyta exhibit developmental cycles is not new. Sauvageau (1897) believed that *Nostoc punctiforme* exhibited an alternation of filamentous and coccoid stages during its life cycle. Moreover, the appearance of floating colonies has prompted investigators to seek a specific mode of development which could account for the formation of these macrostructures composed of regularly oriented microscopic filaments. Usually macrocolony formation is not observed in cultures. For instance, species of *Aphanizomenon* and *Calothrix* exhibit a precise orientation of filaments within a colony when observed in nature, but a disorganized mass of filaments when cultivated *in vitro*. Drewes (1928) observed that hormogonia of *N. punctiforme* formed spiral aggregates on agar.

In cultures of *Nostoc muscorum* A, Allison *et al.* (1937) reported the occurrence of several morphological forms which have been described in this paper. Apparently they did not relate the occurrence of such forms with a regular cycle of development, although they did suggest that the presence of morphological variation might complicate physiological study of this organism. It is not clear from the first accounts of the Allison strain of *N. muscorum*, which apparently was isolated from soil, whether in nature this form exists in colonies. In culture, the tufted appearance of *N. muscorum* A grown in liquid inorganic media or in media containing sucrose suggests a relationship between the developmental cycle at the microscopic level and a primitive morphogenesis at the macroscopic level. The formation of hormogonia with gliding motility is certainly one link between the developmental cycle and macroscopic morphogenesis. It is known from the work of Manten (1948) that the tufts which develop in cultures of *Tolypothrix* are positively phototropic; however, it is not clear whether this phenomenon is linked to phototactic movements of microscopic hormogonia. The microscopic structure of the tufts formed by *N. muscorum* A shows a remarkable organization of the filamentous elements of this presumably primitive organism.

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EXPLANATION OF PLATES

Photomicrographs of *Nostoc muscorum*, Allison strain, in unstained wet mounts of living material.

PLATE 1

Developmental stages of *Nostoc muscorum* A on modified Fogg's agar, incubated at $24 \pm 2^\circ$ at 150 ft.-candles.

Fig. 1. Hormogonia. $\times 1200$.

Fig. 2. A hormogone in which the intercalary cells are dividing in a plane parallel to the axis of the trichome. The terminal cells are differentiating to become terminal heterocysts. $\times 1100$.

Fig. 3. Hormogonia forming the aseriate stage. $\times 1200$.

Fig. 4. New filament formation. $\times 1200$.

Fig. 5. Filament elongation and the formation of hormogonia. $\times 300$.

Fig. 6. In the presence of 1 % glucose, heterocysts are elongated and the formation of hormogonia is inhibited. $\times 1500$.

Fig. 7. Streams of hormogonia migrating on the surface of agar. $\times 1250$.

Fig. 8. Aggregation of hormogonia in spiral forms. $\times 250$.

PLATES 2 and 3

Morphological features of cultures grown in modified Fogg's basal medium at $24 \pm 2^\circ$.

Fig. 9. Filamentous morphology of *N. muscorum* A grown in illuminated cultures on basal medium. Stagnant cultures after 3 weeks of incubation at a light intensity of 200 ft.-candles. $\times 750$.

Fig. 10. Aseriate morphology of *N. muscorum* A grown in complete darkness on basal medium + glucose. The cells are arranged in packets within an enveloping sheath. Intercalary heterocysts are never seen within the packets, although they may be found on the filaments if their differentiation has already begun in the light. Stagnant culture after 6 weeks incubation in complete darkness. $\times 400$.

Fig. 11. Aseriate morphology exhibited when hormogonia produced in illuminated cultures are transferred to media containing glucose and incubated in complete darkness. By growing *Nostoc muscorum* A for 2 weeks in the light with agitation, hormogonia are formed abundantly. When inoculated into tubes containing basal medium + 1% (w/v) glucose and incubated in complete darkness, the intercalary cells of the originally motile trichomes divide slowly to produce packets of large cells enclosed in a retaining sheath. The terminal cells do not divide but differentiate as terminal heterocysts. Intercalary heterocysts are found in such preparations as a result of differentiation which has originated in the light, since intercalary heterocysts are never found within the aseriate packets which represent the growth in the dark. Incubated for 4 weeks in the dark. $\times 700$.

Fig. 12. A preparation similar to that shown in fig. 11, but incubated in darkness for 10 weeks. The large, slowly dividing cells have continued their aseriate mode of growth. $\times 1200$.

Fig. 13. Material grown in the dark for 4 weeks, then placed in light of 150 ft.-candles for 3 days. The aseriate cells have assumed a filamentous arrangement with the production of intercalary heterocysts. The differentiation of intercalary heterocysts is coincident with filament expansion, as may be noted by comparing the relatively 'unopened' filaments at the lower right of the figure with those at the left and above. $\times 620$.

Fig. 14. A preparation similar to that shown in fig. 13. The expanding filaments, newly derived from the aseriate stage, reflect the fact that each cluster of filaments originated from one cell of the original filament. Compare with fig. 3. $\times 880$.

Fig. 15. Four weeks dark growth followed by 4 days in the light. Filament expansion and dissolution of the sheath have advanced to a point where discrete filament clusters are no longer seen. $\times 880$.

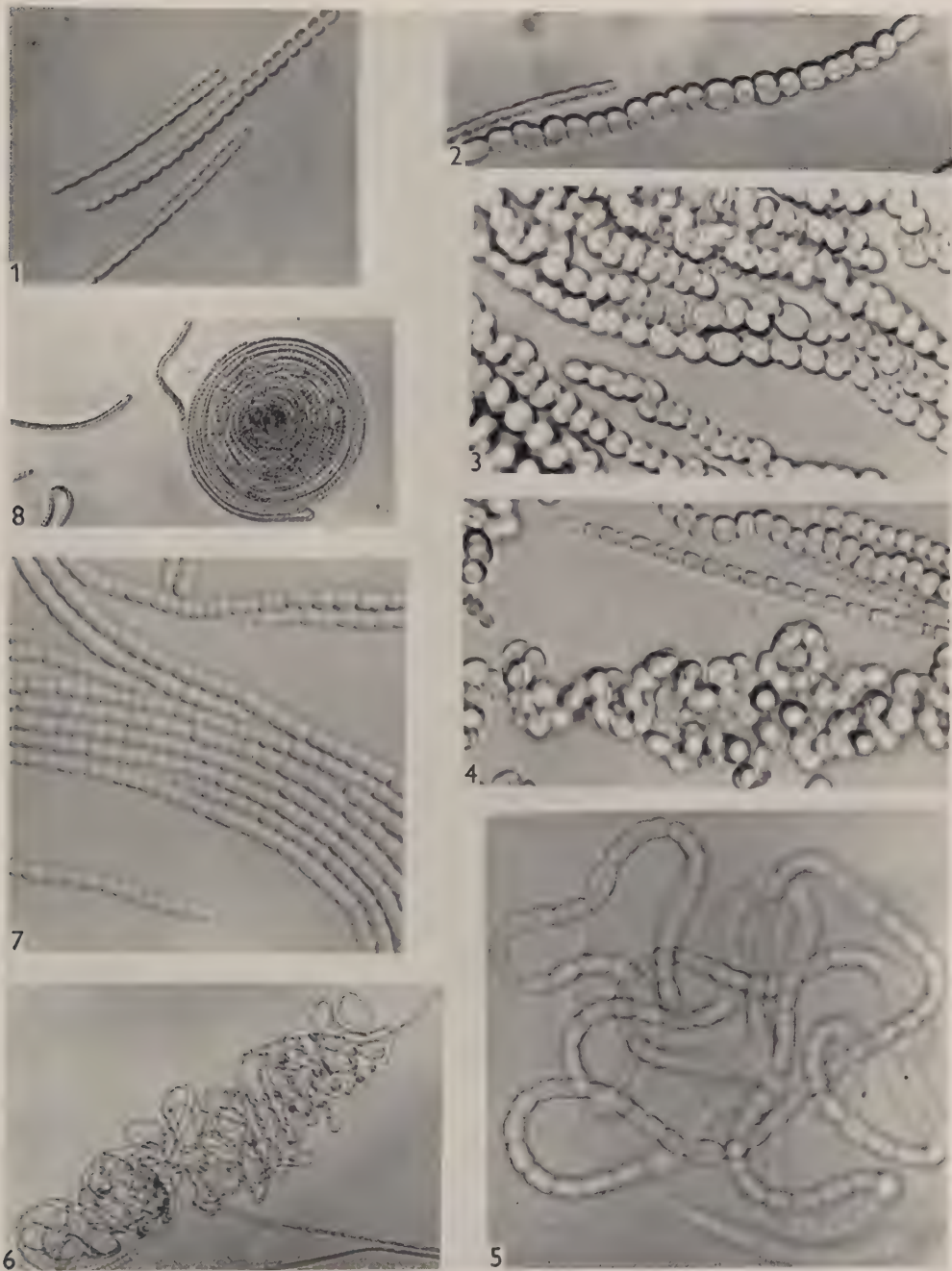
Fig. 16. Four weeks dark growth followed by 4–5 days in the light. Hormogonia begin breaking away from the intercalary heterocysts, which now have a colourless, empty appearance in contrast to their former green colour. The hormogonia are characterized by elongated cells and a distinctly tapered appearance of the terminal cells. The heterocysts often assume a shrivelled appearance, a few minutes after the hormogonia are released. $\times 880$.

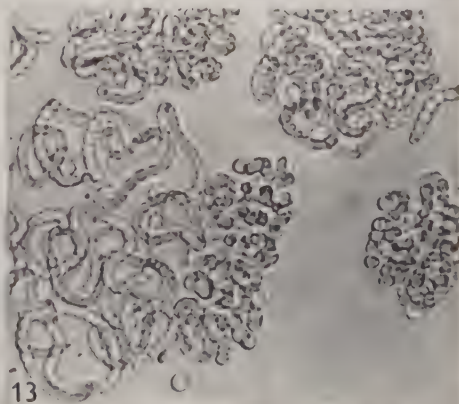
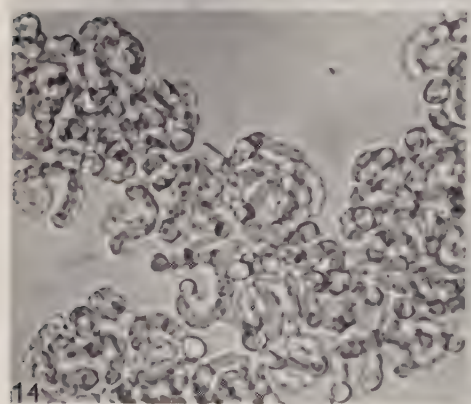
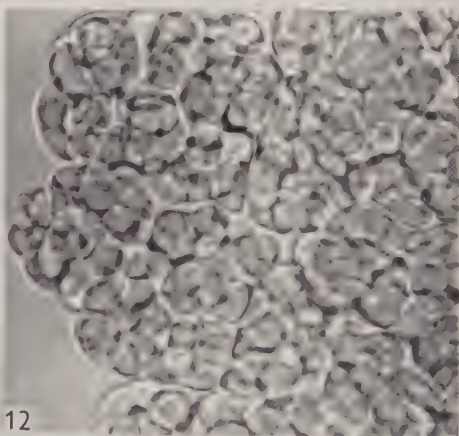
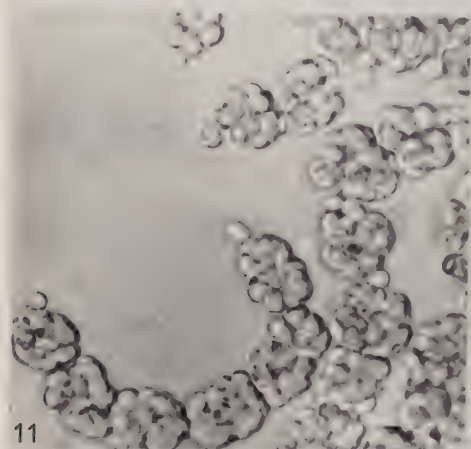
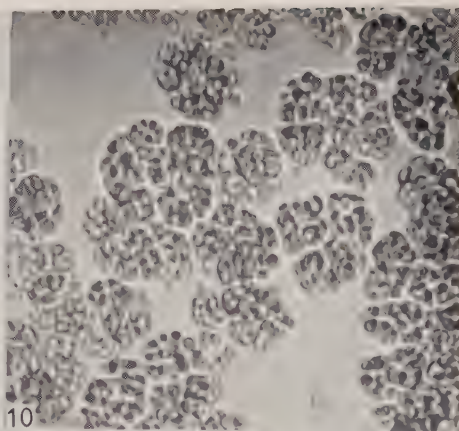
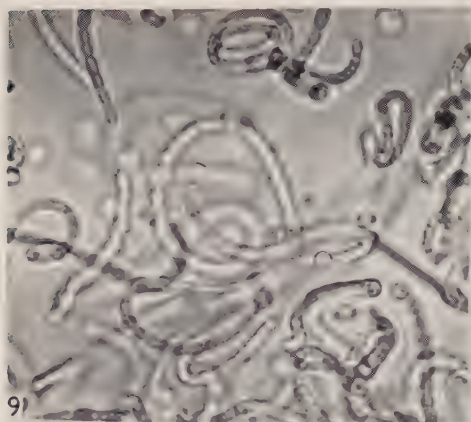
Fig. 17. A preparation similar to that described under fig. 16. The filaments are seen just prior to breaking, and appear to be under tension. Breakage is actually accompanied by a sudden movement as if tension were abruptly released. $\times 750$.

Fig. 18. Four weeks dark growth followed by 5 days in the light. Such cultures consist of masses of motile hormogonia and deposited heterocysts. $\times 750$.

Fig. 19. Four weeks dark growth followed by 12 days in the light. Cultures containing glucose (but not sucrose) cease the formation of hormogonia, possibly owing to the formation of some inhibitory metabolic product. Long filaments are formed with prominent intercalary heterocysts. $\times 875$.

Fig. 20. Further development of material with the 'glucose type of morphology' mentioned above. The heterocysts elongate and often assume a flask-shaped appearance. Filament breakage at one side of the heterocyst may take place, leading to the formation of trichomes with an oscillating movement. $\times 1450$.







Cultivation and Serial Transfer of the Slime Mould, *Dictyostelium discoideum* in Liquid Nutrient Medium

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(Received 20 December 1960)

SUMMARY

A method is described for the routine cultivation and serial passage of the slime mould *Dictyostelium discoideum* in liquid medium in association with *Aerobacter aerogenes*. The growth rates of several strains have been measured under these conditions.

INTRODUCTION

The cellular slime moulds grow by feeding upon bacteria. Although they can be maintained in axenic culture (Bradley & Sussman, 1952), the difficulty of preparing the medium, the slow growth rate and the low yield of organism preclude the use of such cultures for ordinary purposes. In the past, the slime moulds have been cultivated exclusively on solid media. Investigators have thereby been faced, in performing many kinds of experiment, with serious technical difficulties which could have been avoided were it possible to cultivate these organisms in liquid media and to achieve reproducibly high growth rates and yields of organism. It was observed that *Dictyostelium discoideum* would grow luxuriantly in static liquid culture in association with a chromogenic pseudomonad of unknown species (Sussman, 1956*a*). However, the excessive slime production and attendant clumping by the bacteria made it difficult to obtain reproducible growth curves or to harvest the slime mould free from the bacterial associate. It was also observed that incubation of *D. discoideum* in a concentrated suspension of washed *Aerobacter aerogenes* in buffer permitted adequate growth and serial passage of the slime mould (Sussman, 1956*a*). Since then, a detailed procedure has been worked out by Gerisch (1960) under conditions similar to those just mentioned but with *Escherichia coli* as the associate bacterium at a concentration of 10^{10} bacteria/ml. A yield of 10^7 myxamoebae/ml. (0.8 mg. dry weight) and a doubling time of 3 hr. were achieved. Unfortunately, cultivation of myxamoebae with concentrated suspensions of pre-grown bacteria in buffer has certain basic defects. The task of harvesting and washing the bacteria, if it is to be a routine procedure, introduces a high chance of contamination with airborne bacteria and fungi. In practice, it has been impossible to maintain cultures uncontaminated for even a single passage, at least in our hands. Furthermore, the required concentration of bacteria makes it necessary that one grow, harvest, and wash about 7 l. bacterial culture to prepare 1 l. of slime mould culture. This precludes large-scale cultivation for biochemical experiments. The present communication describes a method which permits the routine cultivation and serial passage in liquid medium of *D. discoideum* in association with *A. aerogenes* and which eliminates the disadvantages noted above.

METHODS

Organisms. Two diploid strains of *Dictyostelium discoideum* (designated RA, H-1) and a haploid derivative (44-14) of strain RA were used. Stock cultures were started from single clone isolates and were maintained by mass plating on SM agar (Sussman, 1951; (g./l.): yeast extract, 1; Bacto-peptone, 10; glucose, 10; K_2HPO_4 , 1.0; KH_2PO_4 , 1.5; $MgSO_4$, 0.5; agar, 20) with *Aerobacter aerogenes*.

Composition of the liquid medium. Liquid medium A contained the following constituents (g./l.): Bacto-yeast extract, 0.5; Bacto-peptone, 5; glucose, 5; KH_2PO_4 , 2.25; $K_2HPO_4 \cdot 12H_2O$, 1.5; $MgSO_4 \cdot 7H_2O$, 0.5; to final pH 6-6.3. The medium was dispensed in 18 ml. lots into 125 ml. Erlenmeyer flasks (cotton-wool plugged) and sterilized by autoclaving at 121°.

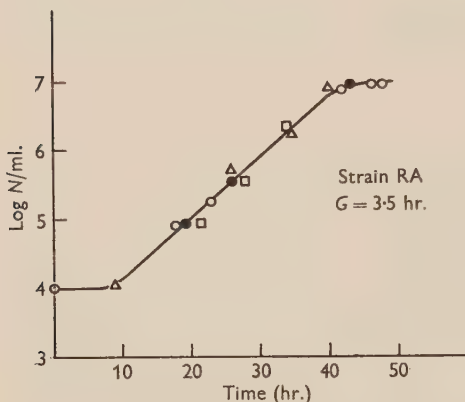


Fig. 1

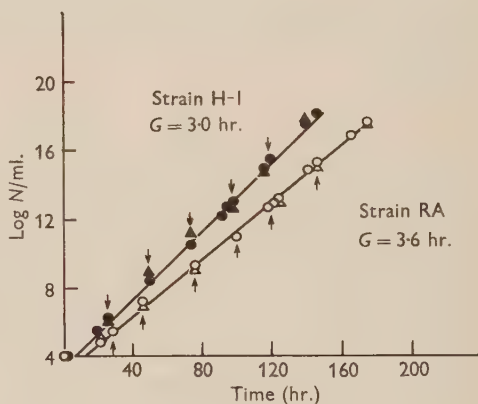


Fig. 2

Fig. 1. The culture cycle of *Dictyostelium discoideum*, strain RA, in medium A. Open and closed circles, squares and triangles represent independent experiments. (N = total no. cells.)

Fig. 2. Sustained exponential growth of *Dictyostelium discoideum* strains RA and H-1 by serial subculture in medium A. Each culture started at a population density of 10^4 myxamoebae/ml. and was allowed to reach 1 to 3×10^6 myxamoebae/ml. before subculture. The arrows designate the times at which the subcultures were made. The ordinate is a cumulative count of organisms, i.e. the last point for one subculture was the zero time point for the next. Circles represent total counts; triangles represent viable counts.

Method of cultivation. One ml. of myxamoeboid or spore suspension at 1 or 2×10^5 organisms/ml. and 1 ml. of a 48 hr. broth (Sussman, 1951) culture of *Aerobacter aerogenes* served as the inocula. The cultures were incubated at 22° on a reciprocating shaker (200 cyc./min.; 1.5 in. stroke).

Growth measurement. Direct counts were made in quadruplicate or octuplicate with a Levy haemocytometer. Viable counts were made from clonal platings (Sussman, 1951) in quintuplicate.

RESULTS

Figure 1 summarizes the data from four separate culture cycles with *Dictyostelium discoideum* strain RA. The inoculum of strain RA consisted of spores taken from 7-day growth plates. (The terminal fruiting stage was reached at 3 days under these

conditions.) A lag phase of about 10 hr. was encountered in the liquid medium. As might be expected, the duration of the lag was found to depend on the age of the inoculum. Spores from older plates displayed a longer lag period, those from younger plates a shorter one, and inoculation of logarithmic phase myxamoebae eliminated it completely. A characteristic exponential phase encompassed about 10 divisions. This indicates a constant generation time and a degree of viability exceeding 95 %, a figure verified by the viable count data shown in Fig. 2. The logarithmic phase generation time, $(t \log 2)/(\log N/N_0)$, was 3.5 hr. In a similar series strain 44-14 (the haploid derivative of RA) yielded a generation time of 3.0 hr., comparable to the values obtained by growth on solid media (Sussman, 1956*b*). The stationary phase is seen in Fig. 1 to have begun at a population density of 8×10^6 myxamoebae/ml., a yield equivalent to that obtained by Gerisch (1960) with pre-grown washed *Escherichia coli* in buffer.

Figure 2 shows the growth of strains RA and H-1 during 7 and 6 serial passages, respectively, in medium A, when inoculated during the logarithmic phase of growth. Exponential growth was maintained over the course of about 40 divisions, representing a 2^{40} dilution of the original protoplasm. The total and viable counts agreed within the limits of random sampling error (± 20 % c.v.). No signs of contamination by airborne bacteria or fungi were seen. In agreement with previous findings (Gerisch, 1960), myxamoebae taken from the liquid culture and dispensed on solid substrata aggregated and fruited normally. The generation time for strain RA was calculated to be 3.6 hr. and for strain H-1, 3.0 hr., a significant difference.

Dictyostelium discoideum can be cultivated in considerably larger volumes of medium A than used in the above experiments. An inoculum of 10^4 myxamoebae/ml. in 1 l. medium A contained in a Fernbach flask and aerated through a sintered glass sparger attained a generation time of 3-4 hr. and a yield of 10^7 myxamoebae/ml.

DISCUSSION

The fact that cellular slime moulds can be grown in the laboratory in liquid media and without special treatment of the bacterial associate raises an important ecological question. In the past, great emphasis has been placed upon the soil habitat in evaluating the ecological position of the Acrasiales (Singh, 1946; Raper, 1951). The conditions for laboratory cultivation in broth are clearly not so critical that they could not be reproduced in nature. A systematic examination of streams and pond water habitats particularly during periods of bacterial bloom might well reveal a new dimension to the natural history of these organisms and might turn up new varieties.

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Pseudomonas—An attempt at a general classification

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SUMMARY

On the basis of the characterization of 126 strains representing 46 different species of *Pseudomonas* and 6 species of *Aeromonas* obtained from different culture collections, a proposal for the taxonomy of the genus *Pseudomonas* is made. By using various old and new diagnostic methods some 90 different morphological and cultural characters, many obtained by modification of methods and media, were investigated. The iodoacetate test was developed to distinguish between oxidative and fermentative utilization of glucose.

Results were evaluated by the use of punched cards and by the method of strips. The relationships between the established species of *Pseudomonas* and *Aeromonas* were shown by using an Adansonian principle and by taxonomic models. On the basis of the results obtained proposals for the definition of the genus *Pseudomonas* and for re-descriptions of *Pseudomonas* species are given and the relations to related genera are shown. In the definition of the genus *Pseudomonas* an attempt to find a more general principle for subdivision within the genus was based on the type of glucose utilization and on the relationships of the species expressed in some other features. This proposal about the taxonomy of pseudomonads is compared with that of Rhodes (1959).

INTRODUCTION

The genus *Pseudomonas* Migula, 1894 is, as compared with some other genera, a very old one. The taxonomy of the pseudomonads has not kept pace with the recent developments of taxonomy in general, and with microbiological taxonomy in particular.

In the literature more than 500 papers deal with the taxonomy of the genus *Pseudomonas* but these are mostly devoted to particular problems, only a few are concerned with the basic aspects. This situation was reviewed and discussed by Seleen & Stark (1943), Haynes (1951), Gaby (1955), Brisou (1958), Rhodes (1959) and others. The result is that more than 140 species are described by Haynes & Burkholder in *Bergey's Manual* (1957) and more than 400 names of *Pseudomonas* species are listed in Krassilnikov's (1949) book; but the species are so ill-defined that the usefulness of the descriptions is doubtful. This is caused first of all by the definition of the genus, which from the time of Migula has been based mainly upon morphology. That the type of flagellation is not an adequate criterion was shown by Bartholomew (1949), Gaby & Free (1953), Sneath (1956*b*), and Rhodes (1958); the pigments produced (Turfitt, 1936; Tobie, 1938) and the serology (Köhler, 1957; Burkholder & Starr, 1948; Naylor, 1954) have not been found suitable bases for taxonomical purposes.

In recent times biochemical and cultural features have been used for the taxonomy of pseudomonads. On this basis an excellent paper concerning the definition of *Pseudomonas fluorescens* and of the genus *Pseudomonas* was published by Rhodes (1959). According to my results the definition of the genus therein is rather narrow, that of the species too broad and sufficient account was not taken of the other species of the genus. One of the main problems in bacterial taxonomy is the lack of a base line or general principle on which species can be founded. What is needed is a system and not only a catalogue of descriptions.

The purpose of the present paper, which is based upon the examination of 125 strains, is to demonstrate that it is possible to develop such a scheme, based on the physiological properties of bacteria in one genus (*Pseudomonas*), and to show the relations between it and other related genera. I shall also make a proposal about the definition of this genus and several main species.

METHODS

A total of 126 different strains (isolates) was used. These strains consisted of 46 different determined species of the genus *Pseudomonas*, 20 undetermined *Pseudomonas* sp. and 6 determined species of *Aeromonas*. Twenty-nine strains were fresh isolates obtained by the author; the other 97 strains were obtained from various culture collections and workers. The origin of the strains comprised strains from: man, animals, plants, soil, fresh water, sea, other sources. Strains were kept as freeze-dried desiccates; for the inoculation of diagnostic media they were maintained and transferred on beef peptone agar slopes or yeast-extract peptone agar slopes.

Diagnostic methods

Cell morphology. Gram reaction, average diameter of organism ($< 1\mu$ or $> 1\mu$), shape of cell ends, occurrence of curved rods, granulation of cytoplasm, presence of fat granules (stained by Sudan III), presence of capsules or extracellular slime (by nigrosin stain and methods used by Rhodes (1958) and Novelli (1953)), and pleomorphism, were determined with organisms from 2- and 7-day cultures grown on meat peptone agar at 28°. Flagella were stained by Bailey's method (Fisher & Conn, 1942) and by Fontana's method (Rhodes, 1958); in several cases electron-microscope examinations were made.

Colonial morphology. The following colonial features were determined with cultures grown on meat peptone agar at 28° for 2 days: form (punctiform, circular or irregular), elevation (flat, raised, convex or umbonate), margin (entire or not entire), surface (smooth or rough), optical characteristics (transparent or opaque).

Appearance on agar slope. On meat peptone agar incubated for 2 days at 28° the intensity of growth and pigmentation was observed; good or scant growth, and colour of pigment and its solubility in the medium were noted.

Glucose utilization. This was determined according to Hugh & Leifson (1953) and by the iodoacetate test, which was developed for this purpose. For the iodoacetate test the following medium was used: Bacto tryptone, 0.1 % (w/v); yeast extract (Difco), 0.1 % (w/v); NaCl, 0.5 % (w/v); K_2HPO_4 , 0.03 % (w/v); glucose, 1.0 % (w/v); 0.2 % (w/v) of aqueous solution of bromthymol blue 1.5 % (w/v); pH 7.2 %. This medium was sterilized by steaming for 30 min. on 3 successive days, and the solution of

mono-iodoacetate, sterilized by filtration, was added so that the final concentration was 10^{-3} M. As control the medium without iodoacetate was used. The medium was inoculated in the normal way, incubated at 28° and the results read for 5 days. The reaction was considered to be positive when acid was produced in both media and negative when acid was formed only in the control tube (without iodoacetate).

Carbohydrate utilization in peptone media. In a medium containing: proteose peptone, 1.0 % (w/v); NaCl, 0.5 % (w/v); carbohydrate, 1.0 % (w/v); bromthymol blue aqueous solution 1/500, 1.2 % (w/v); pH 7.2; acid and gas production were observed for 6 days at 28° . As carbohydrates glucose, galactose, glycerol and mannitol were tested.

Carbohydrates as sole C-sources. These were examined in chemically defined media according to Seelen & Stark (1943) and Dowson (1957) with 1.0 % (w/v) of each carbohydrate, namely glucose, lactose, sucrose, fructose, D-galactose, maltose, D-arabinose, L-arabinose, D-mannose, D-xylose, mannitol, glycerol, inositol, salicin. As a control the medium without carbohydrate was used. The occurrence of an acid reaction and growth were determined during incubation for 7 days at 28° .

Organic acids as C-sources were used in media according to Seelen & Stark (1943) and in Koser's medium in which the citrate was replaced by the salt of another organic acid. In the first medium all organic acids were at 0.3 % (w/v); in the second medium the concentrations were as follows (% w/v): formate, 0.1; oxalate, 0.1; acetate, 0.2; benzoate, 0.3; lactate, 0.1; succinate, 1.0; malonate, 1.0; malate, 1.0; D,L-tartrate, 1.0; citrate, 1.0. Phenol red (1/500) added at 0.6 % (w/v) was used as the pH indicator. Alkaline reaction and growth greater than that in the control medium without C-source was regarded as positive; it was read after 7 days.

Oxidation of phenol, ethanol or gluconate. Ethanol and phenol oxidation was determined in the medium of Dowson (1957) with phenol 0.1 % (w/v) or ethanol 0.5 % (w/v). For the production of acetic acid from ethanol Stanier's method (1947) was used. Gluconate oxidation was determined by the method used by Sneath (1956a). All tests were read after incubation for 7 days at 28° .

Hydrolysis of aesculin, starch, cellulose and pectin. Hydrolysis of starch was tested in 5-day cultures grown at 28° in yeast extract agar containing 0.2 % (w/v) starch, with Lugol's iodine solution; and the aesculin agar plate method (Sneath, 1956a) after 4 days incubation. Ability to attack cellulose was determined in the medium of Alarie & Gray (1947) during incubation for 2 weeks; pectate hydrolysis was observed according to Dowson (1957).

Litmus milk. The reactions in litmus milk were determined after incubation for 2 weeks at 28° .

Phenylalanine, indole, and Voges-Proskauer (VP) tests. The phenylalanine test was made according to the method described in Report (1958); indole was determined by Kovac's reagent added to tryptone medium cultures after 1 week of incubation; the VP test was made by Barritt's method in medium with (% w/v): Bacto peptone, 0.5; dipotassium phosphate, 0.5; glucose, 0.25; galactose, 0.25; after 14 days of incubation.

N-sources and urease production. The utilization of ammonium chloride as N-source was tested in Dowson's medium (1957) with galactose; utilization of nitrite (at 0.05 %, w/v) in Koser's medium with galactose; utilization of urea in Dowson's

medium with urea and phenol red as pH indicator. This latter medium was prepared as a liquid, as well as in solid form. The reactions in all these media were observed for 6 days.

Nitrate reduction to nitrite. For nitrate reduction to nitrite three media were used: (a) Peptone medium of the composition (% w/v): Bacto peptone, 1.0; NaCl, 0.5; KNO₃, 0.1; pH 7.0. (b) Defined medium of Seleen & Stark (1943). (c) Defined medium used by Rhodes (1959). Nitrites were determined after incubation for 7 days by adding Griess reagents and observing gaseous nitrogen in Durham tubes; the result was controlled by the zinc test.

Ammonia formation. Production of ammonia was tested after incubation for 5 days in medium containing 1.5% (w/v) peptone by adding Nessler's reagent.

Decarboxylases. Ability to form decarboxylases of ornithine and lysine, and arginine dihydrolase was tested by Moeller's method (1955); results were determined after incubation for 3 and 5 days.

Proteolytic activity. For liquefaction of gelatin three tests were adopted: (a) yeast extract broth containing 15% (w/v) gelatin; (b) plain gelatin medium, 15% (w/v) gelatin in water; (c) Frazier's method. The results were read after 5 days, 2 weeks, and 5 weeks. Casein digestion was determined in litmus milk after incubation for 14 days and on milk agar plates after incubation for 3 days. For lecithinase C production the method of Knight & Proom (1950) was used.

Lipolytic activity. The lipolytic activity on olive oil was determined by the method of Jones & Richards (1952) as used by Rhodes (1959) and by the method of Bulder (1955). The results were noted after 3 days.

Pigmentation

Pyocyanine. Production of pyocyanine was investigated in the liquid medium of Burton, Campbell & Eagle (1948) and in Gessard's agar (Simon, 1956). After 7 days of incubation at 28° pyocyanine was determined by extraction into chloroform at alkaline pH values (blue) and back into water at acid pH values (crimson) (Wetmore & Gochenour, 1956).

Fluorescein. For the determination of fluorescein the following media were used: (a) beef peptone agar prepared from fresh meat; (b) medium of Georgia & Poe (1931). This pigment was determined by its fluorescence under ultraviolet radiation after incubation for 7 days at 28°.

Melanin and other pigments. The tyrosine medium described by Starr (1946) and by Burkholder & Starr (1948) was used to examine for 'melanin' formation; incubation was for 14 days.

Iodinin and chlororaphin were determined in all the above pigmentation media and on three other media: (a) asparagine, 0.3% (w/v); bipotassium phosphate, 0.05% (w/v); glycerol, 0.5% (w/v); magnesium sulphate, 0.02% (w/v); ferrous sulphate, a trace; pH 7.0, incubation for 14 days at 20°. (b) Bacteriological peptone (Oxoid), 1.0% (w/v); NaCl, 0.5% (w/v); yeast extract (Oxoid), 0.3% (w/v); glucose, 0.5% (w/v); incubation at 20° for 21 days. (c) Malt agar (Oxoid); incubation at 20° for 21 days.

H₂S production was detected by the micro-test used by Rhodes (1959).

Haemolysis. Ability to haemolyse red blood cells was determined after incubation for 3 days on sheep blood agar plates.

Reduction of dyes. Reduction of litmus was examined in litmus milk after incubation for 14 days; methylene-blue reduction was examined by the method of Topley and Wilson's *Principles* (1946, p. 369).

NaCl tolerance. Ability to grow in the media containing different concentrations of NaCl was tested in yeast extract broth and agar with 3.0, 5.0 and 6.5 % (w/v) of NaCl, respectively. Results were noted after incubation for 7 days.

Resistance to pteridine derivative 0/129. Sensitivity to compound 0/129 was tested according to Rhodes (1959).

Resistance to bile was tested on yeast extract agar plates containing 10 or 40 % (w/v) of Bacto ox-gall.

Resistance to antibiotics was tested on agar plates by using tablets containing the following concentrations of antibiotics: penicillin, 10 i.u.; streptomycin, 20 µg.; chloramphenicol, 20 µg.; chlortetracycline, 100 µg.; oxytetracycline 50 µg.

The initial pH value for growth. The test for ability to grow at pH 5.5 and 8.5 was made according to Rhodes (1959).

Thermal death points. Two points were tested, 56° for 10 min. and 42° for 2 days. Both tests were carried out in water baths and broth was used as the medium.

Effect of temperature on growth. The ability to grow at 5°, 10°, 37° and 42° was tested in broth, yeast extract broth and on meat peptone agar slopes. For 5° and 10° a refrigerator and for 37° and 42° water baths were used.

Catalase and oxidase production. Catalase was determined in 2-day cultures grown in broth by adding two drops of hydrogen peroxide. For oxidase formation the Kovacs (1956) method was used.

Inoculation of media

Inoculations were made from 1- or 2-day cultures on agar slopes. For tests in which the degree of turbidity was to be examined, broth cultures were used and inoculation was made by a very small loop; later the method used by Rhodes (1959) was adopted. Each test was repeated at least once during a period of 2 years.

The stability of characters to lyophilization

The influence of lyophilization on some of the features was determined in 90 cultures. The following characters were examined: glucose utilization in Hugh & Leifson's medium, utilization of citrate, formate, acetate, malonate, lactate, tartrate and oxalate in Koser's medium, nitrate to nitrite reduction in peptone medium and Seleen & Stark's medium, liquefaction of gelatin, production of pyocyanine in Burton's and Gessard's media, production of fluorescein in the medium of Georgia & Poe, production of brown pigment on Gessard's medium, growth in 5 % (w/v) NaCl and growth at 37° and 42°. These tests were carried out as described above. The cultures were lyophilized in horse serum containing 7.5 % (w/v) glucose and maintained for 1 year before examination. After this time the ampoules were opened and the tests were repeated.

Recording the results

Two methods were used: punched cards and the 'method of strips'. All tests were coded and according to the code the characters were put on the punched cards. On these cards were also recorded the descriptions found in the literature. This

method was very useful for rapid sorting and determination of correlations between the characteristics. The 'method of strips' was made by recording the features of all strains in a table; this was photographed and a print was cut in strips so that each strip represented one strain. The strips were sorted so that similar strains were put together to form groups. This method was found to be very useful for making a table to show a complex comparison of the strains. The relationship between the strains was also calculated by the method described by Sneath (1957) and expressed by models (Lysenko & Sneath, 1958). The final scheme was obtained on the basis of these methods.

RESULTS

It is impossible to present in a short form all the results obtained. Therefore I propose first to give brief general characterizations of all the strains and then to make proposals for the definition of the genus and a redescription of the main species.

The characters of the strains examined

Morphological characters

Cell morphology. All strains were Gram-negative except *Pseudomonas iodinum* strains. The cells were less than 1μ in diameter, in some strains the ends were pointed; most cells were straight but occasionally slightly curved rods occurred; this was not typical and did not correspond to other features. The presence of granulation of the cytoplasm and the arrangement of cells were not the same for all pseudomonads. The presence of extracellular slime, as described by Rhodes (1958), was a common feature but its intensity might vary, especially in old strains.

Polar flagella occurred in 106 strains, 18 were without flagella and 2 strains, not pseudomonads, possessed peritrichous flagella. The predominant type of flagellation was 1-2 flagella per cell. The importance of flagellation has been discussed by many workers and most recently by Rhodes (1959), and it is generally agreed that the type of flagellation is not an important feature in pseudomonas taxonomy.

Colony morphology. All types and combinations of characters described above occurred in our strains. Many *Pseudomonas aeruginosa* strains were lysogenic. The predominant type of colony was circular or irregular, transparent and smooth. These characters could not be correlated with other features and their taxonomic importance seemed to be very limited.

Agar slant. All strains grew very well.

Cultural and biochemical characters

Types of glucose utilization. In Hugh & Leifson's medium glucose was utilized under anaerobic conditions by 25 strains but late and indistinct reactions were observed in 17 other strains. This test was useful only as a rough guide and did not give such accurate results as the iodoacetate test.

Iodoacetate test. Acid, and sometimes gas, in the control medium but not in iodoacetate medium was produced by only 9 strains; 4 of them were aeromonads, the remaining 5 cultures were not pseudomonads. Fourteen strains did not produce acid in either medium but these strains produced a slight turbidity in iodoacetate medium. 103 strains produced acid in both media, but in 2 strains obtained as *Aeromonas* sp. gas was not produced in iodoacetate medium, which indicates that

these strains had two pathways by which glucose was utilized, oxidative and glycolytic.

The iodoacetate test was found extremely useful for differentiation between oxidative and fermentative utilization of glucose and was valuable for distinguishing between *Pseudomonas* and *Aeromonas* species. It was, however, difficult to state to what extent the glycolytic fermentation was inhibited. This test gave better results than that used by Hugh & Leifson, but we should use carefully the terms 'oxidative or fermentative metabolism' as, even though the glycolytic pathway is blocked by iodoacetate, we know nothing about the other pathways by which glucose may be utilized. The absence of the Embden-Mayerhof system was demonstrated in different pseudomonads (Norris & Campbell, 1949; Campbell & Norris, 1950; Entner & Stanier, 1951; Entner & Doudoroff, 1952; Gibbs & DeMoss, 1954), but on the other hand only in *P. saccharophila* could glucose be utilized in five different ways (Palleroni & Doudoroff, 1957).

The use of well studied enzyme inhibitors, such as dinitrophenol, sodium azide and others, may be useful for better distinguishing the principal metabolic systems, which might be used for a better classification.

Carbohydrate utilization in peptone media. In peptone medium glucose was utilized by 108 strains, galactose by 103 strains, glycerol and mannitol by 29 strains. Gas was produced only by the aeromonads. Other carbohydrates were attacked only by the strains of *Pseudomonas synxantha* and aeromonads.

Carbohydrates as the sole C-source. The utilization of carbohydrates in both media was practically the same, but the use of growth as an indicator of utilization was made difficult because, as described by Rhodes (1959), slight growth might occur in the control tube without C-source. For this reason, only those tubes were classified as 'having growth', in which there was a marked difference in turbidity between the control and test. The frequency of the reactions in 126 strains was expressed as acid reaction growth only: galactose, 125/1; glucose, 84/10; xylose, 74/17; glycerol, 72/14; mannose, 77/21; mannitol, 77/9; fructose, 68/26; L-arabinose, 62/24; D-arabinose, 33/26; inositol, 38/24; sucrose, 20/10; salicin, 16/49; maltose, 14/69; lactose, 3/6.

Organic acids as the sole C-source in Koser's medium were utilized by 126 strains as follows, the results being expressed as the number showing an alkaline reaction/growth only: citrate, 110/6; malonate, 85/20; formate, 81/9; benzoate, 62/21; acetate, 49/40; lactate, 38/9; succinate, 35/6; tartrate, 15/39; oxalate, 3/24; and malate, 4/25. Seleen & Stark's medium gave generally slightly different results; the main differences were in, % positive in Koser: % positive in Seleen & Stark, acetate, 71:56; lactate, 37:79; tartrate, 43:67; and oxalate, 21:0. The differences could be explained by different concentrations of organic acids in both media; similar differences were found by Rhodes (1959).

The cultures gave practically identical results in citrate, malonate, and formate before and after lyophilization, an increased number of positive reactions in acetate and tartrate, but a decreased number in lactate after drying. It was found that the initial pH of the medium played an important role. More positive results were obtained in media with initial pH 6.5 or 6.0 than at pH 7.0. As was said in connexion with the previous tests, the colour changes of the indicator was important for standard checking of the tests.

Oxidation of phenol, ethanol and gluconate. In 126 strains the results (expressed as strong positive:weak positive) were: phenol (12:24), ethanol (41:0) and gluconate (86:4). The ethanol liquid medium gave sharper results than ethanol agar.

Hydrolysis of aesculin, cellulose and pectin. Starch was hydrolysed by 8 strains of which only one was a pseudomonad; cellulose and pectin were not attacked. In aesculin positive reactions were given by 35 strains; weak positive by 7 strains.

Litmus milk. After 14 days' incubation in litmus milk, of 126 strains tested 20 strains gave a strongly alkaline reaction, 1 weakly alkaline, 6 strains acidified and 20 strains produced curd. Peptonization and litmus reduction is given under casein digestion and reduction of litmus.

Phenylalanine, indole and VP tests. The phenylalanine test was positive only once in an aeromonad. The indole test was positive only in 3 aeromonad cultures. The Voges-Proskauer reaction was strongly positive in 19 strains and weakly positive in 1 strain. Most of these strains were classified later as *Pseudomonas synxantha*.

N-sources and urease production. Nitrites were utilized strongly by 73 strains and weakly by 23 strains, ammonium chloride strongly by 48, weakly by 60, and urea strongly by 47 and weakly by 18 strains. Urease was formed by 30 strains. For urease better results were obtained in the liquid medium, because the solid medium gave a higher percentage of uncertain results.

Nitrate reduction. In 3 media the results in 126 strains were as follows, results expressed as number of strains producing NO_2/N_2 : in peptone medium, 194/46; in Selen & Stark's medium, 97/41; in medium used by Rhodes, 82/43. The reduction was influenced by lyophilization so that the ability to reduce nitrates in both media was increased in total, but some strains lost this feature. The results were also influenced by the concentration of nitrates and by the viscosity of the medium. Some strains which gave negative results in the previous media gave weakly positive results in the medium with 0.01% KNO_3 + 0.3% agar; the strains did not produce the same results when the concentrations of nitrates and agar were varied.

Ammonia formation from peptone. Only 2 strains, neither pseudomonads nor aeromonads, did not form ammonia; the remaining 124 strains formed ammonia.

Decarboxylases. Arginine dihydrolase was produced strongly by 90 strains, weakly by 5 strains; ornithine decarboxylase strongly by 15 strains, weakly by 4 strains; lysine decarboxylase strongly by 15 strains, weakly by 4 strains. As will be shown later the arginine test was found to be very useful for pseudomonad taxonomy.

Proteolytic activity. Gelatin liquefaction occurred in 96 strains and the results were practically the same in all media. Strains which did not liquefy gelatin in broth or by Frazier's method were also negative in plain gelatin medium. The speed and the form of liquefaction was variable and seemed not to be important for taxonomy. This characteristic was relatively stable as only 7 strains lost it when the strains were freeze-dried and kept for 1 year.

Casein digestion occurred in 91 strains tested in litmus milk and in 82 on milk agar. The difference was perhaps caused by the different length of incubation.

Lecithinase was formed by 47 strains.

Lipolytic activity. Olive oil was decomposed by 92 strains and 31 strains grew without marked lipolytic activity; 3 strains did not grow.

Pigmentation

Pyocyanin. In the 2 media used pyocyanin was produced by 26 strains on Gessard's medium and by 24 strains in defined medium. This character was stable, as only 2 strains lost it and 1 gained it after the strains were preserved as freeze-dried cultures.

All the strains producing pyocyanine were later found to be *Pseudomonas aeruginosa*.

Fluorescin. In defined media fluorescin was produced by 35 strains but on beef-peptone agar by 71 strains. This difference could be explained by the fact that the method for determination of fluorescin is not standardized (cf. Elliot, 1957), and with different media gives different results. Nevertheless, the better results were obtained in media prepared from fresh meat than from dried commercial products. The differences were caused by the age of cultures, because by lyophilization fluorescin formation was lost in 20 % strains in defined medium and in 22 % on agar medium.

Melanin and other pigments. In defined tyrosine medium none of the strains formed melanin, but some of the strains produced fluorescin or pyocyanin. A brown pigment soluble in the medium occurred often on beef-peptone agar and on Gessard's agar. The intensity of pigmentation was variable and was influenced by the length of incubation.

Iodinine was formed only by *Pseudomonas iodinum* strains on malt agar. Chlororaphin occurred only in some cultures of *P. chlororaphis* in defined medium but this feature was very variable and did not occur in all the strains of this species. *P. aureofaciens* produced orange or yellow pigment corresponding with the description of Haynes *et al.* (1956).

The strains that produced other pigments on different media are described below. One strain of *Pseudomonas pseudomallei* produced slight yellow pigment, a strain of *P. rubescens* formed red pigment as did some of the strains later classified as *P. synxantha* or *Aeromonas salmonicida*. The best pigmentation occurred on milk agar, egg yolk agar or in gelatin after longer incubation at 20°.

Other culture and physiological properties

H₂S production. Using the micromethod, hydrogen sulphide was produced by 40 strains after 4 hr. incubation and by 23 strains after 15 hr.

Haemolysis. Strong haemolysis was caused by 31 of 126 strains and 16 showed a weaker reaction.

Reduction of dyes. Methylene blue was reduced by 25 strains within 2 hr. and by 25 strains within 24 hr. The taxonomic significance of the test was doubtful as it predominated in aeromonads and in *Pseudomonas aeruginosa* and did not correlate with other properties.

Litmus was reduced by 100 strains and this feature correlated with casein digestion.

NaCl resistance. All strains grew in medium with 3 % NaCl, 107 with 5 % NaCl, but only 69 in 6.5 % NaCl. This feature was not influenced by lyophilization.

Resistance to pteridine derivative 0/129. Only the strains of *Pseudomonas fragi*, *P. rubescens* and *P. denitrificans*, as they were later classified, were inhibited by this compound.

Bile resistance. In the medium with 10% bile all strains except 1 of *Pseudomonas rubescens* grew; with 40% bile 2 non-pseudomonads did not grow. Some strains formed an opalescent precipitate surrounding the growth but this feature did not correspond with any other feature.

Antibiotic resistance. The sensitivity of the strains to antibiotics was as follows, expressed as strong/weak: penicillin, 1/1; streptomycin, 68/24; chloramphenicol, 40/48; chlortetracycline, 27/6; and oxytetracycline, 33/6. The strain highly sensitive to penicillin was not a pseudomonad, the one weakly sensitive was an aeromonad. *Pseudomonas aeruginosa* strains were mainly sensitive to chloramphenicol and streptomycin, weakly or not sensitive to the tetracycline antibiotics. In other strains the sensitivity was very variable.

The initial pH for growth. Most of the strains produced distinct growth at pH 5.5 and at 8.5. Only 15 strains did not grow at pH 5.5 and 4 at pH 8.5. The usefulness of these tests was found to be limited, as pseudomonads were able to change the pH on the alkaline side quickly. For this test buffered media should be used.

Thermal death point. Thirty-four strains were sensitive to exposure at 56°/10 min., but this property did not correspond with any other feature. Exposure at 42°/2 days gave better results. Twenty-nine strains which were not able to grow at 42° survived the exposure. This test in combination with the ability to grow at 42° was valuable for distinguishing *Pseudomonas aeruginosa* from other pseudomonads.

Effect of temperature on growth. All strains grew well between 18° and 30°. At 42°, 34 strains grew in broth; in other media the results were similar but solid media in the incubator produced uncertain results. The best method was to use liquid medium incubated in a water bath.

At 37° growth occurred in 106 cultures, at 10° in 84, and at 5° in 56 cultures. In this case, too, clearer cut results were obtained in liquid medium. The inability to grow at 5° and at 10° correlated with the growth at 42° in *Pseudomonas aeruginosa* cultures. Ability to grow at 42° was not affected by the lyophilization and was considered stable. Growth at 37° was an unstable feature; while primary isolation could not grow in subculture, many strains became adapted to grow at this temperature and most strains from collections grew at 37°. For taxonomic purposes the ability to grow at 5° and 42° could be a valuable characteristic.

Catalase and oxidase formation. In my series of strains an oxidase positive reaction was detected in 100 cultures; negative reactions occurred in some non-pseudomonads, in strains later classified as *Pseudomonas synxantha*, in one strain of *P. pseudomallei* and in *P. iodinum*. This test was very useful for the taxonomy of pseudomonads.

Only 4 strains did not form catalase; 2 were not pseudomonads, 1 was an aeromonad and 1 was the culture of *Pseudomonas pavonacea*.

When the results of features examined are compared as a whole with those obtained by Seleen & Stark (1943) or by Rhodes (1959), differences are found. The smaller differences can be explained by the different strains examined; other authors mostly used fresh fluorescein-producing isolates and in this respect their strains were more homogeneous than mine. The marked differences which could not be explained only by this difference occurred mostly in the tests on utilization of carbohydrates or organic acids as C-sources. The results of Rhodes (1959) were on the average 25% higher than mine, but marked differences were in lactose (7.2%

in my series against 90% in Rhodes), in D-arabinose (46·7/80%) and sucrose (23/60%). Discrepancies may be due to the different criteria used for evaluating the results. Rhodes used turbidity, whereas in my work only the change of pH was counted as a positive reaction; turbidity greater than the control was marked as an uncertain reaction. Where a dense turbidity occurred without pH change, it was scored as a late positive reaction. In my experience sharper results could be obtained by using indicators, and the standardized inoculum was not so important, which made the test more suitable for routine diagnostic purposes.

Having the characters of each strain, the next step was to determine if the named strains corresponded with their original descriptions and to choose representative cultures. Except where the originals were available, the descriptions given in *Bergey's Manual* (1957) and in Krassilnikov's book (1949) were used and were supplemented by data in papers published in the two last decades. Even though the descriptions of species were mostly very general, vague or incomplete, and the determination made according to them could be doubtful, the revision and clarification of the taxonomy had, in default of better characterization, to be based on them.

It was found that out of the total number of 106 strains obtained as named cultures, representing 40 species of *Pseudomonas* and 4 species of *Aeromonas*, 58 strains, including 30 *P. aeruginosa* could be taken as representative, 25 strains might be classified as typical; furthermore 17 strains did not correspond to their previous designations and 3 of them could not be taken as pseudomonads or aeromonads.

The taxonomic division of the strains

The work was divided into four steps: (1) to divide the strains in groups according to their similarities, (2) to find the relations between the groups and express them by Adansonian principles, (3) to find the correct names for the groups, and (4) to define the genus and the species. I propose to give only the main points and notes, the detailed results being demonstrated by tables and figures.

The whole series of strains was divided by the sorting methods described into three main groups (the number of strains in the groups is given in the brackets).

(A) A group of 94 strains was divided in two subgroups. The first (a) contained strains later classified as *Pseudomonas aeruginosa* (33 strains), atypical *P. aeruginosa* strains (3), *P. aureofaciens* (2), *P. chlororaphis* (2), *P. aeruginosa*-*P. fluorescens* intermediate strains (1), *P. fluorescens* (9), *P. putida* (8), *P. fluorescens*-*P. putida* intermediate (8) and *P. ovalis* (1).

The variation of characters was least in *Pseudomonas aeruginosa* and the strains were easily distinguished from the others. Closely related to *P. aeruginosa* were the strains classified as *P. aureofaciens* and *P. chlororaphis*. These species were the most biochemically active within this subgroup. The remaining strains formed a series characterized by great variability mainly in carbohydrate utilization, organic acid utilization and in proteolytic activity. To divide them on the basis of the individual characters was impossible. Similar difficulties were demonstrated by many other workers, e.g. Clara (1934), Seelen & Stark (1943), Stanier (1947), Rhodes (1959), and thus the great number of different descriptions of *Pseudomonas* species might be explained. Therefore for the differentiation of these strains two more general criteria were chosen: utilization of carbohydrates as a unit and proteolytic activity. In this way it was possible to distinguish two species: *Pseudomonas*

fluorescens and *P. putida*. The first was characterized by the low activity in utilization of carbohydrates and high proteolytic activity; in the latter these properties were reversed. Between these extremes some intermediate forms occurred. *P. ovalis* was selected as an example of the less reactive species.

The second subgroup (b) was divided into 11 species, later classified as *Pseudomonas pseudomallei* (2 strains), *P. diminuta* (2), *P. stutzeri* (1), *P. denitrificans* (1), *P. iodinum* (1), *P. atlantica* (1), *P. rubescens* (1), *P. pavonacea* (1), *P. geniculata* (1), and *P. taetrolens* (1, and 2 intermediate).

Because strains were few in number it was not possible to establish the range of variation of these species and thus the definitions of the species are of limited value. But many of the strains were holotypes and it was necessary to determine their taxonomic position as there was some relation between them and the species of the first subgroup. The characteristics of these species, which were obtained by the comparison of individual strains, each taken as a unit, are given in the Table 1 and the full descriptions are given at the end of this paper.

(B) The second group was composed of 12 strains later classified as *Pseudomonas synxantha*, 2 strains labelled as *Aeromonas* spp. and 4 strains identified as aeromonads. With these strains it was more convenient to determine the acid production from carbohydrates in peptone than in the defined medium because of the wide range of variation which occurred in the latter medium.

The *Pseudomonas synxantha* subgroup consisted of a very homogeneous group of strains in which each strain possessed no more than three atypical characters from those listed in Table 2. Strains labelled as *Aeromonas* spp. were very closely related to *P. synxantha* but the formation of gas indicated their relations to aeromonads.

The aeromonads, represented here by 4 species, were compared and it was found that they could be distinguished from pseudomonads by the lack of oxidative utilization of glucose as indicated by the iodoacetate test.

The characterization of these species is given in Table 2 and *Pseudomonas synxantha* is defined at the end of this paper.

(C) The last group was composed of 12 different strains which could not be listed in any of the previously described groups and their taxonomic position among pseudomonads was also doubtful. I did not regard these strains as *Pseudomonas* species and they are not described here.

The relationship between the species

The methods used by Sneath (1957) and Lysenko & Sneath (1958) were used to show the relations between species. The S values calculated from Tables 1 and 2 are given in Tables 3 and 4. The models are shown on Pl. 1.

As may be seen the grouping described above was fully confirmed. There are close relationships between species *Pseudomonas aeruginosa*, *P. aureofaciens* and *P. chlororaphis*, between *P. fluorescens*, *P. putida* and *P. ovalis*, and between the species of *Aeromonas*. On the basis of these relationships and on the characters of species the definition of the genus was developed. The general principles used to formulate the definition of the genus given at the end of this paper need explanation here.

The central group of the genus (see scheme in Fig. 1) is connected via *Pseudomonas taetrolens* with *P. synxantha* which is the connecting link to the genus *Aeromonas*. This main line is first of all characterized by the change from the fully

Table 1. Short characterization of species of *Pseudomonas* and *Aeromonas* liquefaciens

Common features of all species (see exceptions in Notes)	Gram					Starch				Glucose pep. med.				
	Glucose + iodoacetate	Ornithine decarboxyl.	Indole	Lipase		Glucose + iodoacetate	Ornithine decarboxyl.	Indole	Lipase	Glucose pep. med.	Galactose def. med.	Catalase	Oxidase	NaCl 5%
	-	+	-	-	or x	-	+	-	-	+	+	+	+	+
						VP								
						Bile 40 %	VP	Glycerol pep. med.	Mannitol pep. med.					

Table 2. Short characterization of the species of *Aeromonas* and *Pseudomonas* synxantha
For comparison the characteristics of *P. aeruginosa* and *P. taetrolens* are given.

Gas from carbohydrates				+	+	+	+	+
42°	+			+				
37°	+	+	+	+	+	+	+	+
10°		+	+		d	+	+	+
5°		+	d			+		+
Litmus red.	+	+	+	+	+	+	+	+
H ₂ S			+	+	+	+	+	+
Casein	+	x	+	x	+	+	+	+
Gelatin	+		+		+	+	+	+
Lysine decarb.			d	+				
Nitrate red.	+	+	+	+	+	+	+	+
Urease	+							+
Formate	+	+	+	+	+	+	x	
Aesculin	d	+	+	+	+	+		+
Gluconate	+		x	+	+	+	+	
In defined medium	Maltose			+	+	+	+	+
	Lactose				+		d	
	Saccharose			+	+		+	
	Inositol			+	+			
	Fructose			+	+	+	+	+
	Mannose		+	+	+	+	+	+
	Xylose	d	+	+	+	d	+	x
Malonate	+	x	x	+	x	x		
Citrate	+	+	+	+	+	x	+	
Oxydase	+	+		d	+	+	+	+
Catalase	+	+	+	+	+	+	+	+
In peptone medium	Mannitol		+	+	+	+	+	+
	Glycerol		+	+	+		+	+
	Glucose	d	+	+	+	+	+	+
	Galactose	+	+	+	+	+	+	+
VP		x	+	+	x	+	+	
0/129 sensit.								
NaCl 5 %	+	+	+	+	+			
Bile 40 %	+	+	+	+	+	+	+	+
Starch						+	+	+
Lipase	+		+	x	+	+	+	+
Indole						+	x	+
Arginine decarb.	+	+		d		+	+	+
Ornithine decarb.		+	+		+			
Glucose + iodoacetate	+	+	+	+				
Gram								
A <i>P. aeruginosa</i>								
B <i>P. taetrolens</i>								
C <i>P. synxantha</i>								
H <i>Aeromonas</i> sp.								
D <i>A. salmonicida</i>								
E <i>A. hydrophila</i>								
F <i>A. liquefaciens</i>								
G <i>A. punctata</i>								

+ = positive, - = negative, x = weak positive or uncertain, d = + or -.

Table 3. *S* values of *Pseudomonas* spp.

Table 3 was derived from Table 1 as follows: for a particular comparison between 2 species, $S = n/(n_s + n_d)$, where n_s is the number of features for which they are both positive, and n_d is the number of features for which one is positive and the other negative. The entries +, (+) and × in Table 1 were counted as positive, — as negative and d as NC (no comparison made).

1 <i>P. aeruginosa</i>	100
2 <i>P. aureofaciens</i>	78, 100
3 <i>P. chlororaphis</i>	79, 97, 100
4 <i>P. aer.</i> — <i>P. fluor. inter.</i>	79, 90, 96, 100
5 <i>P. fluorescens</i>	72, 79, 86, 91, 100
6 <i>P. fluor.</i> — <i>P. put. inter.</i>	61, 68, 72, 73, 94, 100
7 <i>P. putida</i>	73, 75, 80, 83, 78, 80, 100
8 <i>P. ovalis</i>	53, 57, 61, 61, 77, 84, 81, 100
9 <i>P. pseudomallei</i>	77, 88, 92, 91, 79, 72, 86, 69, 100
10 <i>P. denitrificans</i>	61, 60, 60, 60, 59, 63, 65, 62, 72, 100
11 <i>P. stutzeri</i>	68, 68, 63, 58, 68, 70, 52, 58, 62, 75, 100
12 <i>P. iodinum</i>	61, 65, 71, 62, 59, 59, 65, 56, 72, 67, 55, 100
13 <i>P. fragi</i>	55, 59, 58, 54, 69, 81, 66, 74, 62, 77, 60, 54, 100
14 <i>P. diminuta</i>	78, 76, 69, 69, 62, 56, 61, 65, 74, 53, 70, 57, 55, 100
15 <i>P. atlantica</i>	56, 60, 56, 57, 56, 50, 50, 48, 59, 46, 60, 41, 48, 73, 100
16 <i>P. paxtonacea</i>	49, 38, 38, 39, 48, 45, 50, 48, 38, 46, 42, 33, 50, 38, 42, 100
17 <i>P. rubescens</i>	55, 60, 55, 57, 64, 57, 46, 48, 57, 71, 78, 48, 61, 61, 60, 44, 100
18 <i>P. geniculata</i>	55, 62, 59, 58, 62, 55, 44, 48, 62, 50, 71, 50, 54, 61, 74, 48, 74, 100
19 <i>P. taetrolens</i>	62, 72, 76, 70, 68, 66, 58, 52, 70, 63, 60, 47, 60, 62, 52, 35, 52, 56, 100

Table 4. *S* values of some *Pseudomonas* and *Aeromonas* spp.

Table 4 was derived from Table 2 in the same way as Table 3 was from Table 1

A <i>P. aeruginosa</i>	100
B <i>P. taetrolens</i>	56, 100
C <i>P. synxantha</i>	52, 69, 100
H <i>Aeromonas</i> sp.	56, 59, 82, 100
D <i>A. salmonicida</i>	57, 63, 80, 72, 100
E <i>A. hydrophila</i>	48, 63, 75, 69, 75, 100
F <i>A. liquefaciens</i>	47, 53, 66, 68, 69, 88, 100
G <i>A. punctata</i>	42, 53, 56, 52, 59, 79, 82, 100

oxidative pseudomonad to the fully fermentative aeromonad pathway of glucose utilization. The relationships between the species of the central group are shown by the *S* values in Table 3, where the figure 100 indicated identity. The relation between *P. aeruginosa* and *P. taetrolens* lies in the presence of arginine dihydrolase, oxidase, and in the inability to produce acid from inositol, fructose and maltose in peptone medium (cf. Table 2); relations to *P. synxantha* are seen in acid production from glycerol, mannose and mannitol in peptone medium in the presence of ornithine decarboxylase and in slight production of acetoin.

The relationships between *Pseudomonas synxantha* and the species of *Aeromonas* are best seen in the strains labelled as *Aeromonas* spp. which showed both fermentative and oxidative metabolism of glucose, i.e. glucose is utilized with acid production in the medium with or without iodoacetate, gas is formed only in the absence of iodoacetate.

The most closely related species of *Aeromonas* to *Pseudomonas* is *A. salmonicida*; the similarity with *P. aeruginosa* and *P. taetrolens* may be seen in indole, starch,

glycerol, oxidase, inositol, malonate, growth at 42° and pigmentation; the relationship with *P. synxantha* is based on VP, glycerol, mannitol, maltose and ornithine.

The other species of *Pseudomonas* are considered to be transitions between the central group to other genera. The relationship between *P. geniculata* and *Acetobacter* was pointed out by Steel & Walker (1957). The sensitivity to the pteridine derivative shown by *P. fragi*, *P. rubescens* and *P. denitrificans* might indicate a relationship with *Vibrio* (cf. Shewan, Hodkiss & Liston, 1954; Rhodes, 1959). The lack of activity in carbohydrates and organic acids in connexion with weak arginine dihydrolase activity of *P. pavonacea* could indicate a relationship to *Alcaligenes*

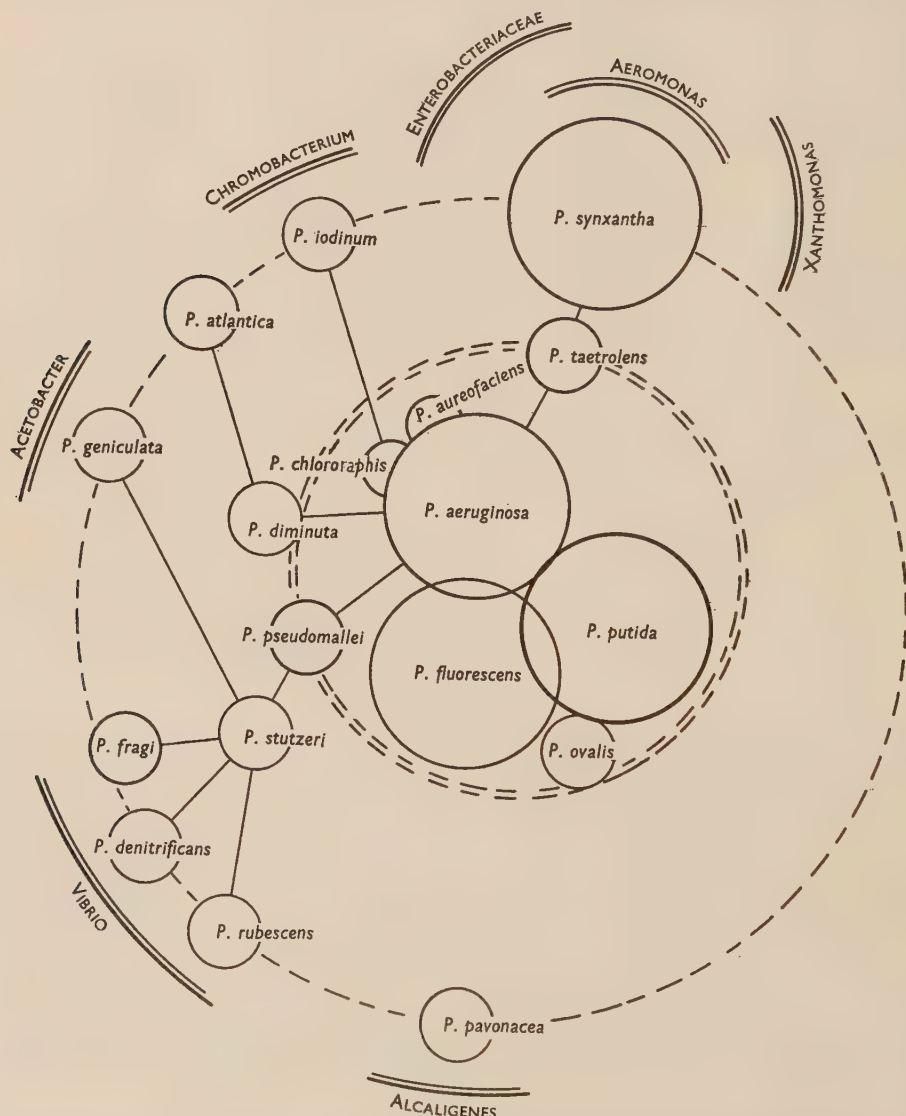


Fig. 1. The relationships between the species of *Pseudomonas* and between the genus *Pseudomonas* and related genera. ---, the boundaries of the genus *Pseudomonas*; ==, the boundaries of the central group. For details see text.

(cf. Sherris, Shoesmith, Parker & Breckon, 1959). The similarity between *P. iodinum* and *Chromobacterium* as well as the relationship between *P. aeruginosa* and *P. pseudomallei* may be seen from the paper by Sneath & Cowan (1958). A relationship between *Aeromonas* and *Serratia* (Enterobacteriaceae) was reported by Stevenson (1959) and some features of *Xanthomonas*, *Cloaca* and *Serratia* spp. are very similar to *P. synxantha*.

In some cases in redescribing species the synonymy was developed partly on cultures which corresponded to the original descriptions, and in other cases on the original descriptions which could not be supported as cultures were not available. According to the *Bacteriological Code* (1958, Rule 9d), when a type culture is not available, characterization depends on a published description and logically, therefore, it is possible to state the synonymy by comparing descriptions. I think that the taxonomy of a group may be simplified and many ill-defined species eliminated in a legitimate way. It is obvious that this solution must be subjective, because the identifications based on an ill-defined description will be doubtful, but any other solution could be just as subjective and the only criterion is convenience.

While establishing the synonymy a more general nomenclatural problem appeared. Some of the previously described species should have been classified as intermediate strains, e.g. occurring between *Pseudomonas fluorescens* and *P. putida* but in the *Bacteriological Code* there is no direction to cover such a situation. There are at least three possibilities: (1) to list them under one of the species, but this is inadvisable as their descriptions do not fully correspond to the definitions of any species; (2) to take them for organisms of uncertain position, but this is not correct, because their taxonomic position can be found; (3) the third possibility is that a new species or subspecies should be created for them, but this also is not correct, because between these newly created species further intermediates will occur and logically new species should be created *ad infinitum*. I think that the existence of intermediate forms must be recognized and should be taken into consideration in the *Bacteriological Code*.

DISCUSSION

In this paper I have tried to find criteria for the more general classification of the genus *Pseudomonas*. An attempt has been made to develop a grouping which is not merely a catalogue of strains but a system having general rules.

My results can be compared in many details with the views and results of other authors, such as Haynes & Burkholder (1957) in *Bergey's Manual* and Rhodes (1959).

The monograph of Brisou (1958) is characterized by the limited number of characters used for species definition without knowledge of the range of variability; therefore, many species are described.

The recent work of Rhodes (1959) in most ways corresponds to the modern requirements of taxonomy. Some of the differences between her work and mine can be explained by the use of different strains and different methods of reading reactions in some media. In addition, differences in the general conceptions and in the evaluation of results, may be summarized as follows: (1) The description of the genus given by Rhodes was based on the examination of isolates which had been selected according to their production of 'characteristic green-yellow water-soluble fluorescein' but this feature was not included in the final definition of the genus or

of the species *Pseudomonas fluorescens*. (2) The genus definition, based on the definition of one species, was narrow, and it was not clear if other species, e.g. the type, *P. aeruginosa*, or other related species, corresponded to it. (3) In the definition the relationship to other related taxa was not taken into consideration.

The last point is important in basic taxonomy and in the past has not received the attention it merits. The definition of a higher taxon, e.g. genus, is not simply the sum of the features common to the lower taxa, just as the bacterial cell is not only the simple sum of enzymes. This additive concept has been common in microbiology and is, I think, a practice that has kept our taxonomy in the stage of catalogues and hindered the development of a system. The characterization of species as the sum of the characters common to all strains, is analogous to defining a forest as the simple sum of trees in it.

On the other hand, my results fully confirm those of Rhodes that the strains classified by her as *Pseudomonas fluorescens*, which in my scheme correspond to the species *P. fluorescens*, *P. putida* and the co-intermediates, have a very high range of variability and that the species must have a broader basis. It is a matter of convenience whether they should be established as two species or as one. I think that it is better to distinguish two species, as *P. fluorescens* was described as gelatin-positive, and to establish *P. putida* for gelatin-negative strains. This feature was correlated to some extent with the carbohydrate utilization. As the strain, which was proposed by Rhodes as a neotype for *Pseudomonas fluorescens*, can be classified as an intermediate between *P. fluorescens* and *P. putida*, a new neotype for *P. fluorescens* was selected.

Finally I should like to emphasize that this taxonomy is only an attempt to show how a more general system might be developed. The taxonomic position of some species is not clear and further knowledge may necessitate a change of their position; moreover, the main principle used here may be found to be of general application and not specific for pseudomonads. But the main point I wish to make is the possibility that a more general scheme in which the intermediate forms can be placed should be found so as to prevent the repeated changes in the taxonomy and nomenclature.

A PROPOSED CLASSIFICATION OF THE GENUS *PSEUDOMONAS*

Genus *Pseudomonas* Migula, 1894, nom. cons.

Syn.: *Chlorobacterium* Guillebeau, 1890.

The genus *Pseudomonas* is composed of species of Gram-negative, aerobic, non-sporeforming, rod-shaped bacteria, which are physiologically a dynamic group. In their physiological characteristics the different forms are linked to one another and form a continuously graded series. The genus as a whole is characterized as follows: straight or slightly curved Gram-negative rods, mostly motile by polar flagella. Spores are not formed. The diameter of cells is less than 1μ . Extracellular slime may be formed.

Glucose utilization is not inhibited by iodoacetate in the concentration of 10^{-3}M . Glucose is utilized oxidatively, gas is not formed from carbohydrates.

Grow well in peptone media, most species grow in defined media in which glucose or galactose are the only C-sources and ammonium phosphate the only N-source.

May form pigments which are green, blue-green, yellow, orange, light or dark brown or fluorescent yellow-green; pigments are mostly soluble in the medium.

If acid is produced from glucose and galactose in peptone medium, acid is not formed from mannitol and glycerol (except *Pseudomonas taetrolens* and *P. synxantha*). Citrate is generally utilized as the only C-source. Arginine dihydrolase may be formed, ornithine decarboxylase is not formed (except *P. synxantha*). Lipolytic. Indole is not formed, starch is not hydrolysed (except *P. stutzeri*), acetoin is not produced, cellulose and pectin are not attacked. Phenylalanine test is negative.

Not sensitive to penicillin. Pteridine derivative 0/129 does not act bacteriostatically (except species transitional to the genus *Vibrio*). Ammonia is formed from peptone. Grow in media with 10 and 40% bile and in medium with 3 and 5% NaCl.

Aerobic. Oxidase (except *Pseudomonas synxantha*, *P. iodinum*) and catalase (except *P. pavonacea*) are formed. Growth occurs at 18–30°, some species grow at 5°, others at 42°. Growth at initial pH 5.5–8.5.

Mostly saprophytic, but some species may be pathogenic.

The type species is *Pseudomonas aeruginosa* (Schroeter) Migula, nom. cons.

The principle of the taxonomy within the genus

The taxonomy is based on species-centres, which are defined by all the physiological features so that an individual centre shares some common features but has others that are exclusive. Between the species many intermediate forms may exist. The main line of variation is based on the transition from forms possessing purely oxidative utilization of glucose to forms which both oxidize and ferment glucose.

The central group within the genus consists of species possessing exclusively an oxidative metabolism of glucose, utilizing citrate and malonate as the only C-source, utilizing nitrites and ammonium chloride as N-source, oxidizing gluconate, forming arginine dihydrolase. Lactose is not utilized; they are not sensitive to pteridine derivative 0/129. Oxidase and catalase are formed. To this group belong the species *Pseudomonas aeruginosa*, *P. fluorescens*, *P. putida*, *P. chlororaphis*, *P. aureofaciens* and transitional forms as *P. pseudomallei*, *P. ovalis* and *P. taetrolens* in which some of the above features may be absent. The species are established according to their carbohydrate and nitrogen metabolism, *P. aeruginosa* being the most active species and *P. ovalis* the least.

With this central group are linked further species which are transitional to related genera, such as *Aeromonas* and *Xanthomonas* (*Pseudomonas synxantha*), *Chromobacterium* (*P. iodinum*), *Vibrio* (*P. fragi*, *P. rubescens*, *P. denitrificans*), *Acetobacter* (*P. geniculata*) *Alcaligenes* (*P. pavonacea*). The taxonomic setting of these transitional species is not final, as it depends upon further similar elaboration of the related groups.

Pseudomonas aeruginosa (Schroeter) Migula, 1900, nom. cons.

Syn.: *Bacterium aeruginosum* Schroeter, 1872; *Bacillus pyocyaneus* Gessard, 1872; *Pseudomonas pyocyanea* Migula, 1895.

Cells. Gram-negative rods, spores are not formed, motile by 1–2 polar flagella. Cells form irregular slimy capsules or irregular extracellular slime.

Colonies. On meat peptone agar: circular or irregular, flat, raised or umbonate, margin is entire or irregular, surface smooth or rough, transparent or opaque. Frequently lysogenic.

Agar slope. Good growth. Some strains form pigment which is soluble in the medium.

Pigmentation. Fluorescin, pyocyanine and brown pigment are formed. The production of one of these pigments may be absent. Non-pigmenting strains may occur.

Physiological and cultural characters are given in Table 5.

Some strains are pathogenic for animals, man or insect. Widely distributed in nature (man, animals, plants, water, soil).

Strain CCEB 481 (i.e. Culture Collection of Entomogenous Bacteria, Laboratory of Insect Pathology, Prague) is proposed as neotype.

Pseudomonas aureofaciens Kluyver *et al.* 1956.

This species is closely related to *Pseudomonas aeruginosa*. Pyocyanine and fluorescin and the brown pigment are not formed. An orange or yellow phenazine pigment is formed. The cells possess tufts of polar flagella. Physiological and cultural features are given in Table 5. Occurs in soil.

Strain CCEB 518 (NRRL B-1576) is the lectotype culture.

Pseudomonas chlororaphis (Gignard & Savegeau) Bergey *et al.* 1930.

Syn.: *Bacillus chlororaphis* Gignard & Savegeau, 1894.

This species is closely related to *Pseudomonas aeruginosa*. The cells are motile by tufts of polar flagella. Pyocyanine is not formed, fluorescin is sometimes formed. Some strains can form chlororaphin irregularly.

Physiological and cultural characters are given in Table 5.

Occurs in milk, in insects and perhaps widely distributed in nature.

CCEB 292 (NRRL B-560) is proposed as neotype.

Pseudomonas fluorescens (Flügge) Migula, 1895.

Syn.: *Bacillus fluorescens liquefaciens* Flügge, 1886; *Pseudomonas viscosa* (Frankland & Frankland) Migula, 1895; *P. septica* Bergey *et al.* 1930 (i.e. *Bacillus fluorescens septicus* Stutzer & Wsorrow, 1927); *P. boreopolis* Gray & Thornton, 1928; *P. shuylkilliensis* Chester, 1901 (i.e. *Bacillus fluorescens shuylkilliensis* Wright, 1895); *P. putrefaciens* (Derby & Hammer, 1931), Long & Hammer, 1941; *P. cohaerens* (Wright, 1895), Chester, 1901.

Cells. Gram-negative rods, spores are not formed, motile by polar flagella, cells form irregular capsules or extracellular slime.

Colonies. On meat peptone agar: circular, flat, raised or convex, entire, smooth, transparent.

Agar slope. Good growth; green, yellow-green or slightly brown soluble pigment.

Pigmentation. Fluorescin or slightly brown pigments may be formed. Some strains are without pigment.

Physiological and cultural characters are given in Table 5.

Note. From the number of 14 different carbohydrates listed in the table which are utilized as the C-sources, 3 carbohydrates are utilized with acid production and 3 others without acid.

Widely distributed in nature.

The strain CCEB 488 is proposed as neotype.

Pseudomonas putida (Trevisan) Migula, 1895.

Syn.: *Bacillus fluorescens putidus* Flügge, 1886; *Pseudomonas striata* Chester, 1901 (i.e. *Bacillus striatus viridis* Ravenal, 1896); *P. incognita* Chester, 1901 (i.e. *Bacillus fluorescens incognitus* Wright, 1895; *P. rugosa* (Wright, 1895), Chester, 1901; *P. mildenbergi* Bergey *et al.* 1930; *P. convexa* Chester, 1901 (i.e. *Bacillus fluorescens convexus* Wright, 1895); *P. eisenbergii* Migula, 1900 (i.e. *Bacillus fluorescens nonliquefaciens* Eisenberg, 1891, *P. nonliquefaciens* Bergey *et al.* 1923); *P. ambigua* (Wright, 1895), Chester, 1901 (i.e. *Bacillus ambiguus* Wright, 1895); *P. salopia* Gray & Thornton, 1928.

Cells. Gram-negative, rods with polar flagella, motile. Extracellular slime is formed irregularly.

Colonies. On meat peptone agar: circular, flat, raised, convex or umbonate; entire, smooth, transparent.

Agar slope. Good growth. Fluorescin or brownish pigment may be formed.

Pigmentation. Fluorescin or slightly brown pigments may be formed.

Physiological and cultural characters are given in Table 5.

Note. In contrast to *Pseudomonas fluorescens*, acid is formed from 6 carbohydrates of those listed in the table in the defined medium.

Widely distributed in nature.

CCEB 520 is proposed as neotype.

Pseudomonas ovalis Chester, 1901

Syn.: *Bacillus fluorescens ovalis* Ravenal, 1896.

Cells. Gram-negative, non-sporeforming rods, motile by tufts of polar flagella. Extracellular slime may be formed.

Colonies. On meat peptone agar: circular, flat, entire, smooth, transparent.

Agar slope. Good growth with the formation of yellow-green pigment soluble in medium.

Pigmentation. Fluorescin is formed.

Physiological and cultural characters are given in Table 5.

CCEB 380 (NRRL B-1595) is proposed as neotype.

Pseudomonas pseudomallei (Whitmore) Haynes, 1957

Syn.: *Bacillus whitmori* Stanton & Fletcher, 1921; *Bacillus pseudomallei* Whitmore, 1913; *Malleomyces pseudomallei* Breed, 1939; *Loefflerella pseudomallei* (Whitmore) Gay *et al.* 1935.

Cells. Gram-negative rods, non-sporeforming, motile, by 1-2 polar flagella. Capsules are not present.

Colonies. On meat peptone agar: small, circular, smooth or rough. Yellowish or white with oil-like lustre.

Agar slope. Good growth, some strains grow slowly.

Pigmentation. Yellow pigment not soluble in medium occurs in some strains. In older cultures slight brown pigment develops.

Physiological and cultural characters are given in Table 5.

Note. The acid formation in defined and peptone media with carbohydrates is variable in the sense that young and old cultures give different results. Haemolysis of sheep erythrocytes is very weak or may be absent but human erythrocytes are haemolysed.

Pathogenic for man and animals.

The strain CCEB 472 (NRRL B-12) is proposed as neotype.

Pseudomonas fragi (Eichholz, 1902) Huss, 1907 emend. Hussong *et al.* 1937

Syn.: *Bacterium fragi* Eichholz, 1902.

Cells. Gram-negative, non-sporeforming rods, motile by polar flagellum. Extracellular slime may be formed.

Colonies. On meat peptone agar medium: circular, flat or raised, entire, smooth, transparent.

Agar slope. Good growth, brownish pigment.

Pigmentation. Brownish pigment, soluble in medium, is sometimes formed in cultures.

Physiological and cultural characteristics are given in Table 5.

Strain CCEB 387 (NRRL B-25, ATCC 4973) is proposed as neotype.

Pseudomonas diminuta Leifson & Hugh, 1954

Cells. Gram-negative non-sporeforming rods, motile by polar flagellum. Extracellular slime is formed. In all the cultures slight pleomorphism occurs.

Colonies. On meat peptone agar: circular, umbonate, entire, transparent, smooth.

Agar slope. Good growth.

Pigmentation. In older cultures a brownish pigment soluble in medium occurs.

Physiological and cultural characteristics. See Table 5.

The strain CCEB 513 (NCTC 8545) is the holotype.

Pseudomonas stutzeri (Lehmann & Neumann) Kluyver, 1942

Syn.: *Bacillus denitrificans* Burri & Stutzer, 1895; *Bacterium stutzeri* Lehmann & Neumann, 1896; *Bacillus nitrogenes* Migula, 1900; *Pseudomonas stutzeri* Kluyver, 1942; not *Pseudomonas stutzeri* Migula, 1900.

Cells. Gram-negative rods, non-sporeforming, motile by a polar flagellum. Extracellular slime is formed only rarely.

Colonies. On meat peptone agar: circular, convex, entire, transparent, smooth.

Agar slope. Good growth without pigment formation.

Physiological and cultural characteristics are given in Table 5.

CCEB 522 (NRRL B-927) is proposed as neotype.

Pseudomonas denitrificans Bergey *et al.* 1923.

Syn.: *Bacillus denitrificans fluorescens* Christensen, 1903.

Cells. Gram-negative, non-sporeforming rods, motile by 1-2 polar flagella. Extracellular slime may be formed.

Colonies. On meat peptone agar: circular, flat with an irregular margin, rough, slightly transparent.

Agar slope. Good growth, without pigments.

Physiological and cultural characters. See Table 5.

Occurs in water and soil.

CCEB 525 (NRRL B-1028) is proposed as neotype.

Pseudomonas iodinum (Davis, 1939) Tobie, 1939

Syn.: *Chromobacterium iodinum* Davis, 1939.

Cells. Gram-variable, especially in the young cultures. Non-motile rods without flagella. Extracellular slime is formed very weakly.

Colonies. On meat peptone agar medium: circular, white, convex, entire, smooth, opaque.

Agar slope. Good growth.

Pigmentation. On malt agar after longer incubation at 20° a reddish pigment occurs. The production of this pigment, which is a phenazine derivative, is very variable.

Cultural and biochemical characters are given in Table 5.

The strain NCTC 9742 (CCEB 512) is designated as the lectotype.

Pseudomonas atlantica Humm

Cells. Gram-negative, non-sporeforming rods, motile by polar flagellum. Extracellular slime is rarely formed.

Colonies. On meat peptone agar: very small circular.

Agar slope. Good growth.

Pigmentation. In old cultures brownish pigment soluble in medium.

Cultural and biochemical characters are given in Table 5.

Occurs in sea water.

Note. The strain CCEB 506 (National Collection of Marine Bacteria 301), which was obtained as the type culture, differs in some characters from the original description. In peptone medium it does not form acid from glucose, galactose, glycerol and mannitol, starch is not hydrolysed, acetate and lactate are not used as C-sources, tartrate is used, nitrites are not used as N-sources.

Pseudomonas rubescens Pivnick, 1955

Cells. Gram-negative, non-sporeforming rods, motile by a polar flagellum. Extracellular slime is not formed.

Colonies. On meat peptone agar: circular, convex, entire, smooth, transparent.

Agar slope. Good growth.

Pigmentation. On egg yolk agar and milk agar a purple pigment not soluble in medium is formed.

Cultural and biochemical characters are given in Table 5.

Isolated from mineral oils.

The strain CCEB 519 (NRRL B-1651) is the lectotype.

Pseudomonas pavonacea Levine & Soppeland, 1926

Cells. Gram-negative, non-sporeforming rods, motile by a polar flagellum. Extracellular slime may be formed. Old cells are a little pleomorphic.

Colonies. On meat peptone agar: circular, convex, entire, smooth, transparent.

Agar slope. Good growth and in older cultures a very slightly yellow-green pigment soluble in the medium is formed.

Pigmentation. Very weak and not marked. Might be fluorescein.

Cultural and biochemical characters are given in Table 5.

The strain CCEB 533 is proposed as neotype.

Pseudomonas geniculata (Wright, 1895) Chester, 1901

Syn.: *Bacillus geniculatus* Wright, 1895.

Cells. Gram-negative rods. Spores are not formed. Motile by means of polar flagella. Extracellular slime may be formed. In culture pleomorphic forms may occur.

Colonies. On meat peptone agar: circular, convex, entire, smooth, transparent.

Agar slope. Good growth.

Pigmentation. Brownish pigment soluble in the medium may be formed.

Cultural and physiological characters are given in Table 5.

Strain CCEB 338 is proposed as neotype.

Pseudomonas taetrolens Haynes, 1957

Syn.: *Pseudomonas graveolens* Levine & Anderson, 1932; not *Pseudomonas graveolens* Migula, 1900.

Cells. Gram-negative rods, non-sporeforming, motile by a polar flagellum. Extracellular slime is formed irregularly. In old cultures irregular, pleomorphic filamentous forms occur.

Colonies. On meat peptone agar: circular, convex, entire, slightly transparent, smooth.

Agar slope. Good growth.

Pigmentation. Slightly brown pigment is formed in old cultures. Pigment is soluble in the medium.

Physiological and cultural characters are given in Table 5.

Strain CCEB 381 (NRRL B-14) is the holotype.

Pseudomonas synxantha (Ehrenberg) Holland, 1920

Syn.: *Pseudomonas lachrymans* (Smith & Bryan, 1915), Carsner, 1918; *Bacillus apisepticus* Burnside, 1936 (cf. Landerkin & Katznelson, 1959).

Cells. Gram-negative rods, motile by polar flagella or non-motile. Capsules or extracellular slime not formed.

Colonies. On meat peptone agar: circular, flat or raised or convex, entire, smooth, transparent or opaque.

Agar slope. Good growth.

Pigmentation. Some strains can form brownish or pinkish pigment soluble in the medium.

Cultural and biochemical characters are given in Table 5.

Occurs in insects, on plants, in sea and perhaps widely distributed in nature.

Strain CCEB 293 (NRRL B-780, ATCC 9890) is proposed as the neotype.

Table 5. Cultural and physiological characters of *Pseudomonas* sp.

[illegible]

Nitrate to

	Nitrate to	Source of N	Utilization or oxidation of	Hydrolysis of	VP	Indole	Phenylalanine	Litmus milk	Nitrite N	Ammonium N	Urea N	Urease	NH ₄ from peptone	NO ₂	Nitrogen	Arginine	Ornithine	Lysine	Gelatin	Casein	Lecithinase	H ₂ S	Lipase	Haemolysis
1 <i>P. aeruginosa</i>			(d)	-	-	-	PR	A	+	+	+	d	+	+	d	+	-	-	+	+	+	d	+	+
2 <i>P. aureofaciens</i>			+	-	-	-	PR	O	+	+	+	d	+	+	+	+	-	-	+	+	+	d	+	+
3 <i>P. chlororaphis</i>			+	-	-	-	PR	PRO	+	+	+	d	+	+	+	+	-	-	+	+	+	d	+	+
5 <i>P. fluorescens</i>			+	-	-	-	PR	O	+	+	+	d	+	+	+	+	-	-	+	+	+	d	+	+
7 <i>P. putida</i>			+	-	-	-	PR	PRO	+	+	+	d	+	+	+	+	-	-	+	+	+	d	+	+
8 <i>P. ovalis</i>			+	-	-	-	PR	PRO	+	+	+	d	+	+	+	+	-	-	+	+	+	d	+	+
9 <i>P. pseudomallei</i>			+	-	-	-	PR	PRO	+	+	+	d	+	+	+	+	-	-	+	+	+	d	+	+
13 <i>P. fragi</i>			+	-	-	-	PR	A	+	+	+	d	+	+	+	+	-	-	+	+	+	d	+	+
14 <i>P. diminuta</i>			+	-	-	-	PR	A	+	+	+	d	+	+	+	+	-	-	+	+	+	d	+	+
11 <i>P. stutzeri</i>			+	-	-	-	PR	A	+	+	+	d	+	+	+	+	-	-	+	+	+	d	+	+
10 <i>P. denitrificans</i>			+	-	-	-	PR	A	+	+	+	d	+	+	+	+	-	-	+	+	+	d	+	+
12 <i>P. iodinum</i>			+	-	-	-	PR	A	+	+	+	d	+	+	+	+	-	-	+	+	+	d	+	+
15 <i>P. atlantica</i>			+	-	-	-	PR	A	+	+	+	d	+	+	+	+	-	-	+	+	+	d	+	+
17 <i>P. rubescens</i>			+	-	-	-	PR	A	+	+	+	d	+	+	+	+	-	-	+	+	+	d	+	+
16 <i>P. pavonacea</i>			+	-	-	-	PR	A	+	+	+	d	+	+	+	+	-	-	+	+	+	d	+	+
18 <i>P. geniculata</i>			+	-	-	-	PR	A	+	+	+	d	+	+	+	+	-	-	+	+	+	d	+	+
19 <i>P. taetrolens</i>			+	-	-	-	PR	A	+	+	+	d	+	+	+	+	-	-	+	+	+	d	+	+
<i>P. synzantha</i>			+	-	-	-	PR	A	+	+	+	d	+	+	+	+	-	-	+	+	+	d	+	+

Table 5 (cont.)

	Resistance				Antibiotics				Production of				Growth at				Pigments			
	Growth in medium with				Penicillin				Oxidase				42°				Light brown			
	Bile				Streptomycin				Catalase				37°				Fluorescein			
	NaCl				Chloramphenicol								10°				Other phenazine			
Reduction of					Chlortetracycline								5°				Pyocyanin			
					Oxytetracyclin															
Litmus	Growth at initial pH																			
	Sensitiv. 0/129																			
Methyl. blue	8.5																			
	5.5																			
	40 %																			
	10 %																			
	6.5 %																			
	5 %																			

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EXPLANATION OF PLATE

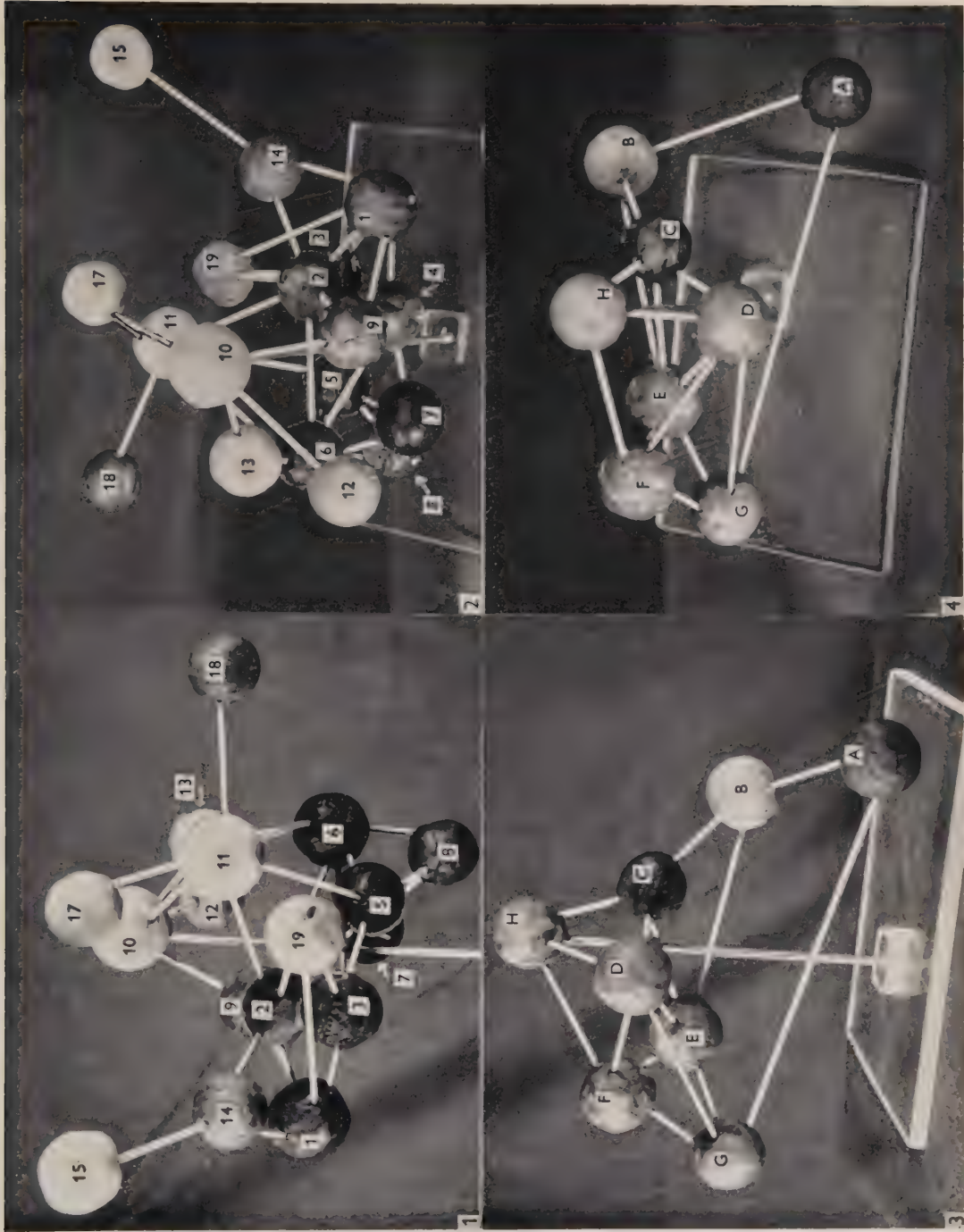
PLATE I

Fig. 1. Taxonomic model of the *Pseudomonas* sp. The model is made according to the S values given in Table 3. The distances (*d*) between species represented by balls are converted from the S values, $d = (1/S) - 1$. The numbers are those used for the species in Tables 1 and 3.

Fig. 2. Taxonomic model of the *Pseudomonas* species: another view.

Fig. 3. Taxonomic model of *Aeromonas* sp. and *Pseudomonas synxantha* and *P. aeruginosa*. Models made according to the S values given in Table 4. The letters are those used for the species in Tables 2 and 4.

Fig. 4. Taxonomic model of the *Aeromonas* sp., *Pseudomonas synxantha* and *P. aeruginosa*: another view.



Lysogeny and Colicinogeny in *Escherichia coli*

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SUMMARY

One hundred and eighty-three strains of *Escherichia coli* were examined for lysogeny and colicinogeny: 40.4 % were neither lysogenic nor colicinogenic; 31.1 % were only colicinogenic; 13.1 % were only lysogenic; 15.2 % were lysogenic and colicinogenic. Though the detection of lysogeny could almost certainly be increased by using a greater number of sensitive indicator strains or by using more sensitive methods, the results of this study suggest: (a) that lysogeny in *E. coli* is more frequent than found in earlier studies (Fredericq, 1952); (b) that lysogeny and colicinogeny are not correlated but occur independently in *E. coli* strains.

INTRODUCTION

Escherichia coli, *Shigella* spp. and some other groups of Enterobacteriaceae, which are often colicinogenic, were found to be rarely lysogenic by Fredericq (1952). On the other hand, salmonellas are commonly lysogenic but very seldom colicinogenic (Fredericq, 1952; Hamon, 1955; Papavassiliou & Samaraki-Lyberopoulou, 1957; Papavassiliou, 1960a; Vassiliadis, Papavassiliou, Glaudot & Sartiaux, 1960). In this study, 183 strains of *Escherichia coli*, comprising 115 freshly isolated strains of unknown serological type, 40 strains of serotype 111:B4 pathogenic for infants, and 28 strains of serotype 70:B80 pathogenic for calves, were examined for both lysogeny and colicinogeny.

METHODS

Organisms examined. Of the strains examined 115 were freshly isolated from cultures made directly from human or animal faeces on MacConkey agar plates. These were examined for lysogeny and colicinogeny within a week of their isolation. Tests on freshly isolated strains were made before the organisms were identified; results with strains of the groups Klebsiella, Cloaca, Citrobacter and atypical strains are not reported here. Forty strains of *Escherichia coli* serotype 111:B4, isolated in this laboratory from faeces of infants were also examined; these had been stored in ordinary media at room temperature for a few weeks. The remaining 28 strains belonged to *E. coli* serotype 70:B80 pathogenic for calves, and were received from Professor H. Fey (Faculty of Veterinary Medicine, University of Berne, Switzerland).

Indicator strains. *Escherichia coli* strain Row, obtained from Professor P. Fredericq (University of Liège, Belgium), was used in testing for colicinogeny. In tests for lysogeny, three strains of *E. coli* were used, namely, B, C and Y 20. All three were

obtained from Professor P. Fredericq. Strain Y 20, a derivative of *E. coli* K 12 sensitive to phage λ , and previously used in tests for lysogeny with this phage (Fredericq & Papavassiliou, unpublished), proved a less sensitive indicator for lysogeny in general than the first two. Strain B was a line of *E. coli* B, the strain used as indicator for the T series of phages; and strain C was a line of *E. coli* C, a strain used as indicator for the presence of phages in water.

When a strain was colicinogenic, it was tested for lysogeny with mutants of *Escherichia coli* B or C resistant to the colicine or colicines which it produced. For instance, *Escherichia coli* B/I (resistant to colicine I) was used in testing strains found to produce colicine I, and *Escherichia coli* B/E+I in testing strains which produced colicines E+I.

Media. Oxoid nutrient broth CM1 and Oxoid nutrient agar CM3 were used in this study. Nutrient broth was distributed in 5 ml. quantities. Nutrient agar plates contained about 40 ml. agar. The soft agar was prepared from Oxoid nutrient broth (5 g. agar in 1000 ml. broth).

Tests for colicinogeny. The double-layer method of Gratia (Fredericq, 1957) was used. Inocula from 24 hr. broth cultures of the strains to be tested were stabbed into nutrient agar with a straight wire, 8 strains/plate. After incubation for 48 hr., the macrocolonies which had developed were sterilized by chloroform vapour, and the indicator strain, *Escherichia coli* Row, was seeded over the whole surface by pouring 0.1 ml. of a 6 hr. broth culture diluted with 0.9 ml. broth and then mixed with 4-5 ml. melted soft agar cooled to below 50° on to the plate. After incubation of the plates for 24 hr., clear zones of inhibition of the indicator could be seen round colicinogenic macrocolonies. The colicine which a strain produced was identified by testing it for activity against a series of mutants of *E. coli* strains Row or K 12 resistant to different known colicines (Fredericq, 1957).

Tests for lysogeny. The indicator strain was grown in 5 ml. broth for 4-5 hr. from an inoculum taken with a 5 mm. loop from a 24 hr. broth culture. The indicator plate was prepared by pouring 0.1 ml. of this culture diluted with 0.9 ml. broth and then mixed with 4-5 ml. soft agar melted and cooled to below 50° on to the agar plate. The plate was then dried by incubation at 37° for 30-45 min. with the lid open. Cultures of the strains to be tested grown for 3-4 hr. at 37° in 5 ml. broth from inocula of 0.1 ml. taken from 24 hr. broth cultures were sterilized by chloroform (Fredericq, 1950). After standing on the bench for at least 30 min., a 5 mm. loopful was placed on the surface of the indicator plate, and the plate again dried for 30-45 min. with the lid open. The plates were examined after 20 hr. incubation at 37°.

With strains which were found to be non-colicinogenic, when plaques due to phage were present they were usually not subcultured. With colicinogenic strains, although colicine-resistant indicators were used, subcultures were made to confirm the presence of phage. No isolations or titrations of phages were made, however, as the object was to detect lysogenic strains, not to isolate new phages.

RESULTS

Of the 183 strains examined, 24 (13.1 %) were lysogenic, 57 (31.1 %) only colicinogenic, 28 (15.2 %) both lysogenic and colicinogenic, and 74 (40.4 %) neither lysogenic nor colicinogenic (Table 1). The total percentage of lysogenic strains (regardless of colicinogeny) was 28.3 %; the percentage of colicinogenic strains was 46.4 %. Table 1 also shows the origins of the strains.

Table 2 shows the types of colicines produced by the colicinogenic strains, and the numbers of these strains which were also lysogenic. No association was observed between lysogeny and the type of colicine produced.

Table 1. *Lysogeny and colicinogeny of 183 strains of Escherichia coli and their origin*

Origin	Strains	Lysogenic	Colicino- genic	Lysogenic and colicino- genic	Neither lyso- genic nor colicinogenic
Adult faeces	94	9	29	13	43
Serotype 111:B4 (infant faeces)	40	6	12	14	8
Animal faeces (rat and mice)	21	7	0	0	14
Professor Fey's strains (calf pathogenic)	28	2	16	1	9
Total	183	24	57	28	74

Table 2. *Occurrence of lysogeny in colicinogenic Escherichia coli*

Source or serotype	Type of colicine produced	No. strains examined	No. lysogenic strains
Adult faeces	E	6	0
	E+I	10	3
	I	26	10
Infant faeces	I	25	14
serotype 111:B4	E	1	0
Calf pathogenic serotype 70:B80	V	3	1
	B	3	0
	E	1	0
	I	6	0
	W	4	0
Total		85	28

The specific serotypes of *Escherichia coli* 111:B4 (pathogenic for infants) and *E. coli* 70:B80 (pathogenic for calves) showed a particular incidence of lysogeny and colicinogeny. From the 40 strains of the serotype 111:B4, 26 (65 %) were colicinogenic, 25 produced colicine I, and one produced colicine E, and 20 (50 %) were lysogenic. From the 28 strains of the serotype 70:B80, 17 (60.7 %) were colicinogenic, but only 3 (10.7 %) were lysogenic. These results indicate that no direct relation existed between colicinogeny, lysogeny and pathogenicity, and also that pathogenic types could be subdivided into several types according to colicine-

production, type of colicine produced, lysogeny and the combinations of these characteristics. This might be of epidemiological importance (Fredericq, Betz-Bureau & Nicolle, 1956; Hamon & Brault, 1959).

DISCUSSION

Lysogeny and colicinogeny in *Escherichia coli* are not associated, but occur independently. The present results are thus similar to those of Nagy (1959) who studied lysogeny and bacteriocinogeny in 100 strains of *Bacillus megaterium*. As he says 'no genetic correlation between the two lethal biosyntheses could be demonstrated and their occurrence was found to be essentially independent'. The incidence of colicinogeny reported in the present study (46.4 %) is higher than the incidence reported by Fredericq (1957), Hamon & Brault (1959) and Papavassiliou (1959, 1960c) in other studies. This is possibly due to the examination of a number of strains of the serotypes 111:B4 and 70:B80, a high proportion of which were colicinogenic.

The incidences of lysogeny and colicinogeny found among the pathogenic serotypes cannot be considered as final: (i) the number of strains examined was small; (ii) at least some of these strains may possibly have been repeated isolations of the same strain. We have not yet typed strains from different laboratories all over the world, but the present results show clearly that even this small number of strains examined fall into several types with regard to their production of colicine, the type of colicine produced and lysogeny. By these characters strains isolated in Athens fall into 5 types and Professor Fey's strains into at least 7 types. The number of these types might possibly be increased by the examination of other characters, for instance sensitivity to colicines or the identification of phages produced.

The most important difference from previous results is the high incidence of lysogeny (28.3 %), which may have resulted from our use of different indicator strains (Fredericq, 1952). The incidence of lysogeny may have been even greater than actually observed, since the detection of lysogeny requires suitable indicator strains, and in addition some phages are sensitive to chloroform. In any case the incidence of lysogeny in *Escherichia coli* reported here is lower than the incidence reported for *Salmonella* (Fredericq, 1952) *Proteus* (Vieu, 1960) or *Bacterium anitratum* (Papavassiliou, 1960b). In the present study, no difference in the incidence of lysogeny was observed between human and animal strains, but human strains are more frequently colicinogenic than animal strains (Papavassiliou, 1960c). *E. coli* B and *E. coli* C detect more lysogenic strains than *E. coli* Y20. When the first two indicator strains were compared, while a number of lysogenic strains gave confluent lysis of B and only discrete plaques on C, only one strain gave confluent lysis of C and only plaques on B.

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Induction of Malic Enzyme and of Oxalacetate Decarboxylase in Three Lactic Acid Bacteria

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SUMMARY

The presence of inducible, DPN-specific malic enzymes have been demonstrated in *Lactobacillus plantarum*, *L. casei* and *Streptococcus faecalis*. Of 30 compounds tested, only L-malate induced the synthesis of malic enzyme in *L. plantarum*.

The possibility that the malic enzymes of these three organisms are bifunctional proteins which have separate sites to decarboxylate oxalacetate has been excluded. In addition to L-malate, three compounds, oxalacetate, acetoacetate and α -keto-glutarate, induce the synthesis of oxalacetate decarboxylase even though they cannot induce the synthesis of malic enzyme. The oxalacetate decarboxylation system consists of an oxalacetate decarboxylase and an oxalacetate permease. Malate can induce the synthesis of only oxalacetate decarboxylase and not oxalacetate permease.

INTRODUCTION

It has been suggested that both malic enzyme activity ($\text{L-malate} + \text{DPN} \leftrightarrow \text{pyruvate} + \text{DPNH} + \text{H}^+ + \text{CO}_2$) and oxalacetate decarboxylase activity (oxalacetate $\xrightarrow{\text{Mn}^{++}}$ pyruvate + CO_2 , not oxalacetate $\xrightarrow{\text{ATP}}$ phosphoenopyruvate + CO_2) of *Lactobacillus plantarum* (*arabinosus*) are located on the same protein (Korkes & Ochoa, 1948). These investigators used the term 'bifunctional protein' for an enzyme protein which can catalyse two separate reactions, which occur at different sites on the same protein. This concept distinguishes a 'bifunctional protein' from a poorly specific enzyme protein (which can accept more than one substrate at the same site). The assignment of the term 'bifunctional' to the malic enzyme of *L. plantarum* had depended upon the observation that acetone-dried preparations of *L. plantarum*, which had been grown in the presence of malate, contained both malic enzyme and oxalacetate decarboxylase activities whilst those grown in the absence of malate contained neither activity. During a study of compounds which might serve as inducers of the *L. plantarum* malic enzyme the possibility that oxalacetate decarboxylase might be independently induced, and so not be part of a bifunctional protein, was investigated.

METHODS

The organisms used were *Lactobacillus plantarum* 17-5 (ATCC 8014); *L. casei* (ATCC 7569); *Streptococcus faecalis* (ATCC 8043). The methods of Nathan (1961) were used for maintenance and preparation of inocula. Organisms to be used for

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induction experiments were grown in 1 l. batches in a modified Wright & Skeggs (1944) medium. For *L. casei* and *S. faecalis* this medium was supplemented with either leucovorin (0.1 $\mu\text{g./ml.}$) or folic acid (0.1 $\mu\text{g./ml.}$) respectively.

For the detection of inducers, organisms were grown for 12 hr. at 36° in 1 l. mass cultures. Potential inducers (1.5 mmole) were added to 50 ml. samples of the 12 hr. culture and the incubation at 36° was continued for a further 3 hr. The organisms were then harvested by centrifugation, washed and resuspended so that a 1/10 dilution of the resultant suspension had an optical density of 0.4 when measured in a Klett–Summerson colorimeter equipped with a no. 42 (420 m μ) filter.

The amount of malic enzyme, or of oxalacetate decarboxylase, synthesized during the 3 hr. incubation period with a potential inducer, was subsequently assessed manometrically. For assay of malic enzyme, the main compartment of each Warburg vessel contained MnCl_2 , 6.0 μmole ; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 6.0 μmole ; KCl, 6.0 μmole ; 1.2 ml. 0.1 M-phosphate buffer (pH 5.6); DL-malate, 0.23 mmole; in the side arm, 0.3 ml. suspension of organisms or acetone-dried organisms (20 mg. dry weight). The final volume was adjusted to 3.0 ml. with distilled water. The enzyme contents of organisms incubated with potential malic enzyme inducers were compared with that of organisms incubated with L-malate and with organisms incubated without addition of potential inducer.

For the assay of oxalacetate decarboxylase, the main compartment of each Warburg vessel contained MnCl_2 , 6.0 μmole ; 1.0 ml. 0.1 M-acetate buffer (pH 4.5); one side arm contained 0.3 ml. cell suspension of organisms or acetone-dried organisms. The final volume was adjusted to 3.0 ml. with distilled water. In all estimations of enzymic decarboxylation of oxalacetate, non-enzymic decarboxylation was deducted. The enzyme content of organisms incubated with potential oxalacetate decarboxylase inducers was compared with that of organisms which had been incubated with oxalacetate and with organisms incubated without addition of potential inducer.

For the detection of permeases, malic enzyme or oxalacetate activity of suspensions of organisms was compared with the activity of an acetone-dried preparation made from the same batch of organisms. It was assumed that acetone-drying would destroy any specific permeability barriers and that substrates would thus be freely diffusible.

RESULTS

Experiments with Lactobacillus plantarum

Malic enzyme

Of 30 compounds tested for ability to induce malic enzyme, only L-malate (which also serves as substrate for this enzyme), induced synthesis of appreciable amounts of malic enzyme (Table 1). There was thus a striking specificity for induction. An illustration of a typical experiment is given in Fig. 1. Compounds which showed more than traces of activity as inducers included as compared with 100% for L-malate: fumarate 3–8, mesaconate 3–10, DL-alanine 7, DL-methyl succinate 3–12, L-tartrate 1–3, D-tartrate 1–3, meso-tartrate 1–3, ketomalonate 1–3, DL- α -methylglutarate 1–3. The apparent activity of fumarate may have been a reflexion of activity of malate produced by the reaction: fumarate \rightarrow malate and not be indicative of activity of fumarate itself.

Acetone-dried preparations made from malate-grown *Lactobacillus plantarum* retained the ability to decarboxylate malate; i.e. the malic enzyme was stable to

acetone-drying (Blanchard, Korkes, Del Campillo & Ochoa, 1950). While not all of the 30 compounds previously tried as inducers were retested, no malic enzyme activity was found in acetone-dried preparations made from organisms grown with

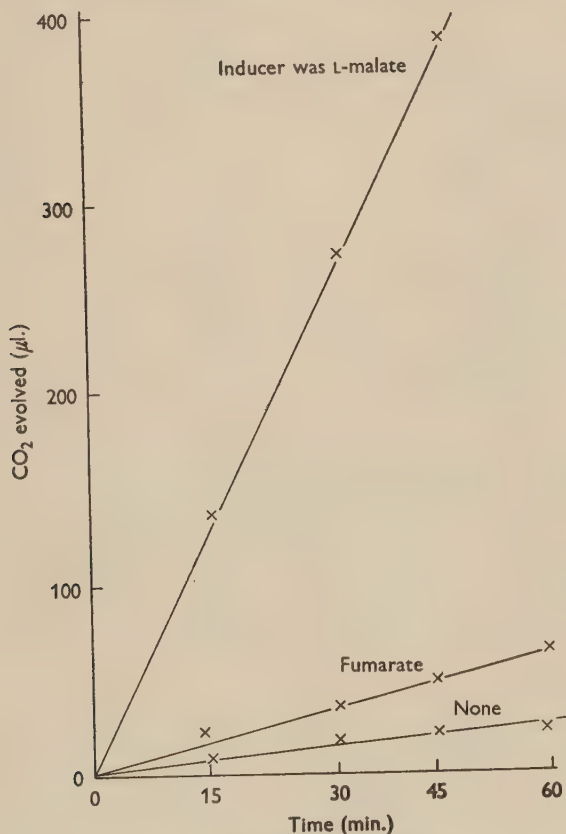


Fig. 1. Malic enzyme content of *Lactobacillus plantarum* grown in media containing potential inducers. Curves represent results obtained with suspensions of organisms which had been grown with different inducers during the final 3 hr. of growth.

oxalacetate, acetoacetate, or β -hydroxybutyrate as potential inducers. Evidence will be presented that these compounds do penetrate the intact cell and in these circumstances it would be possible that they might be able to induce malic enzyme. It is also possible that there is a malate permease whose induction can be demonstrated with intact organisms. Since active enzyme preparations (in which the specific permeability would be destroyed) from organisms grown with these substrates also do not show malic enzyme activity, it seems unlikely that an inducible malate permease exists.

Oxalacetate decarboxylase

This enzyme catalyses the irreversible conversion of oxalacetate to pyruvate. Full oxalacetate decarboxylase activity was shown by intact *Lactobacillus plantarum* grown with oxalacetate or acetoacetate, 90% activity for those grown with α -

Table 1. *Compounds tested for ability to induce synthesis of malic enzyme and oxalacetate decarboxylase in Lactobacillus plantarum*

Compound	Ability to induce synthesis of	
	Malic enzyme	Oxalacetate decarboxylase
L-Malate	100*	100†
Oxalacetate	0	100*
Acetoacetate	0	100
α -Ketoglutarate	0	90
Fumarate	3-8	0
Mesaconate	3-10	N.T.‡
DL-Alanine	7	N.T.
DL-Methyl succinate	3-12	1-3
L-Tartrate	1-3	0
D-Tartrate	1-3	0
meso-Tartrate	1-3	0
Ketomalonate	1-3	N.T.
DL- α -Methylglutarate	1-3	N.T.
β -Hydroxybutyrate	0	1-3

The following compounds could not induce synthesis of either enzyme: D-malate; DL- α -hydroxybutyrate; L-citramalate; DL-citramalate; succinate.

The following compounds could not induce synthesis of malic enzyme and were not tested for ability to induce oxalacetate decarboxylase: thiomalate; chloromalate; maleate; malonate; D-lactate; α -methyl- α -hydroxybutyrate; glutarate; β -hydroxy- β -methyl-glutarate; itaconate; tartronate; pyruvate.

* Ability of compounds to induce enzyme synthesis were compared with these reference compounds.

† Induces synthesis of the decarboxylase but not the permease.

‡ Not tested.

ketoglutarate and 1-3% activity for those grown with either DL-methyl succinate or β -hydroxybutyrate (Tables 1 and 2). It was surprising to find that intact *L. plantarum* which had been grown with L-malate did not decarboxylate oxalacetate, especially since Korkes & Ochoa (1948) had based their argument for the presence of a bifunctional protein on data which showed that acetone-dried preparations from malate-grown organisms contained oxalacetate decarboxylase, i.e. that ability to decarboxylate malate and oxalacetate were simultaneously induced. We therefore compared the oxalacetate decarboxylase activity of intact *L. plantarum* with that of acetone-dried preparations and found that while intact organisms grown with malate did not decarboxylate oxalacetate, acetone-dried preparations made from the same organisms did show oxalacetate decarboxylase activity. These data could be explained by the occurrence of an inducible oxalacetate permease.

Oxalacetate permease

Whole organisms of *Lactobacillus plantarum* grown with L-malate were unable to decarboxylate oxalacetate whereas acetone-dried preparations from the same batch of organisms decarboxylated oxalacetate readily. That is, L-malate induced the synthesis of malic enzyme and oxalacetate decarboxylase but not the permease which permits the penetration of oxalacetate into the intact organism.

Organisms grown with oxalacetate did not decarboxylate malate, nor did acetone-dried preparations made from these organisms. Thus oxalacetate induced oxalacetate

Table 2. *Specificity for induction of oxalacetate decarboxylase, oxalacetate permease and malic enzyme in Lactobacillus plantarum and Streptococcus faecalis*

	Ability to induce		
	Oxalacetate		Malic enzyme
	Decarboxylase	Permease	
Oxalacetate	+	+	0
Acetoacetate	+	+	0
α -Ketoglutarate	+	+	0
L-Malate	+	0	+

permease and oxalacetate decarboxylase but not malic enzyme, and malate induced oxalacetate decarboxylase and malic enzyme but not oxalacetate permease. It therefore seems highly unlikely that the malic enzyme and oxalacetate decarboxylase are part of one 'bifunctional protein'. Indeed the evidence for such a bifunctional protein, i.e. consistent simultaneous induction used as a criterion by the earlier workers (Korkes & Ochoa, 1948), has been shown to be incomplete by the experiments presented here, since the evidence of Korkes & Ochoa rested only on induction by malate and not on induction by oxalacetate.

Our results, summarized in Table 2, show that L-malate can induce malic enzyme and oxalacetate decarboxylase but not oxalacetate permease whilst oxalacetate, acetoacetate and α -ketoglutarate can induce oxalacetate decarboxylase but not malic enzyme. Competence as inducer of oxalacetate permease seems to depend upon the presence of a free keto group. From these data, one may conclude that (a) oxalacetate decarboxylase and malic enzyme are separate enzymes and not part of a bifunctional protein, (b) the oxalacetate decarboxylation system of intact *Lactobacillus plantarum* consists not only of an inducible decarboxylase but also of an inducible permease.

Experiments with Streptococcus faecalis and Lactobacillus casei

Illustrations of results are made with data from experiments done with *Streptococcus faecalis*. Since the same results were obtained from duplicate experiments done with *Lactobacillus casei*, these data are not reproduced here.

The presence of inducible malic enzymes in *Streptococcus faecalis* and *Lactobacillus casei* was established by growing the organisms in the modified Wright & Skeggs (1944) medium, or in this medium + DL-malate (0.1%, w/v). Suspensions of the resultant organisms which were grown in the presence of malate showed malic enzyme activity; thus the *S. faecalis* and *L. casei* malic enzymes were inducible.

The coenzyme requirements of the *Streptococcus faecalis*, *Lactobacillus casei* and *L. plantarum* malic enzymes were found to be DPN-specific. The experiment illustrated in Fig. 2 shows results obtained with acetone-dried *S. faecalis*. Because of imperfect removal of the endogenous coenzyme during acetone-drying, it was possible to demonstrate inhibition of this residual activity by added TPN (Fig. 2).

A survey of potential inducers of malic enzyme revealed that the specificity for the *Streptococcus faecalis* malic enzyme was at least as strict as for the *Lactobacillus plantarum* malic enzyme. The presence of inducible oxalacetate decarboxylase and oxalacetate permease and the separate induction of the oxalacetate system

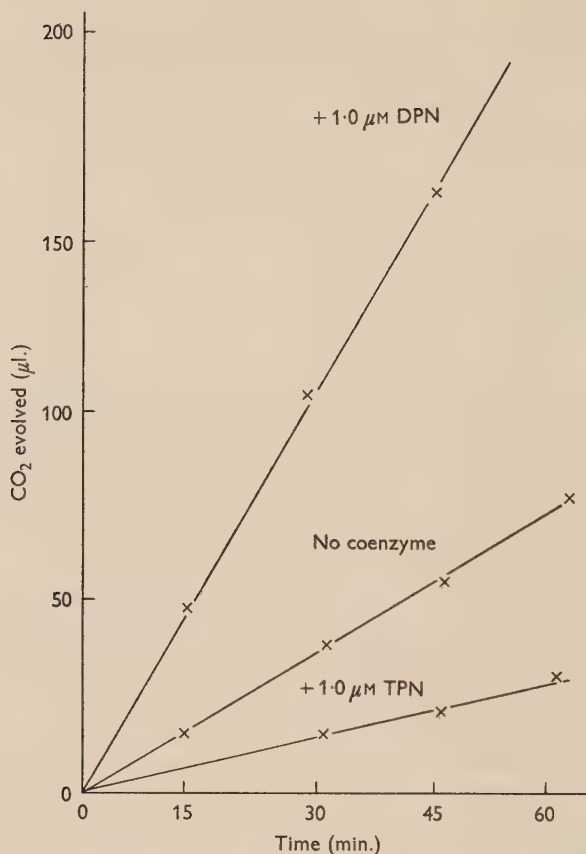


Fig. 2. Coenzyme specificity of the *Streptococcus faecalis* malic enzyme. Enzyme preparation used was acetone-dried cells.

and the malic enzyme described above for *L. plantarum* was also found to occur with the *S. faecalis* and *L. casei* enzymes.

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The Effect of Environment on the Replication of Poliovirus in Monkey Kidney Cells

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SUMMARY

Poliovirus can complete its maturation in monkey kidney cells suspended in semi-solid agar. Infectious particles and the progeny from each infected cell diffuse through the semi-solid agar to form a single plaque in an underlying monkey kidney cell monolayer. Using this technique, the effect of environment on the multiplication of poliovirus in singly infected monkey kidney cells was studied. Under optimal conditions of pH and temperature (pH 7.0-7.4, 37°) one-step growth curves indicated that over 95 % of the adsorbed virus particles went into eclipse; that the eclipse phase lasted 3-4 hr.; that the increase in infective virus was exponential and that a yield of 700-1400 infectious particles/infected cell was obtained in 12-14 hr. After about 10 % of the total progeny had formed, the cells began to release virus. Under suboptimal conditions of pH or temperature, the eclipse stage was prolonged. At pH 6.0-6.5 it lasted for 6 hr. and at pH 5.0-5.5 for more than 9 hr. At 25° virus particles were adsorbed and went into eclipse, with the exception of some particles which remained infectious and could be neutralized by antiserum. Thereafter the eclipse stage was strikingly prolonged, and replication proceeded on raising the temperature to 37°.

INTRODUCTION

One-step growth curves have been described for poliovirus multiplying in suspensions of HeLa cells (Dulbecco & Vogt, 1955) and R.K. cells (Lwoff & Lwoff, 1960) and in monolayers of monkey kidney cells (Howes & Melnick, 1957; McLaren, Holland & Syverton, 1959) and HeLa cells (Maassab, Loh & Ackermann, 1957; Darnell, 1958; McLaren *et al.* 1959). The present paper reports an investigation of the effect of environmental conditions on the eclipse phase of poliovirus in monkey kidney cell suspensions studied by a technique in which not only the virus but also the infected cells could be titrated.

METHODS

Virus. A suspension of Type I poliovirus, Parker strain, grown in trypsin-dispersed monkey kidney cultures, was used.

Antiserum. This was prepared in rhesus monkeys; a 1/250 dilution of serum neutralized 30 infectious particles of the poliovirus suspension.

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Monkey kidney monolayers. These were prepared from rhesus monkeys (Youngner, 1954) and cultivated in 6 cm. Petri dishes.

Media. The following media, containing 100 μ g. streptomycin/ml., 100 i.u. penicillin/ml. and 70 units (E. R. Squibb) mycostatin/ml. were used: (a) Hanks's solution containing 0.5 % (w/v) lactalbumin hydrolysate and 5 % (v/v) calf serum, adjusted to pH 7.0-7.4 with 5 % (w/v) NaHCO_3 solution. (b) Medium 199 supplied by Microbiological Associates, Bethesda, Maryland, U.S.A. (c) Agar overlay (Youngner, 1956).

Solutions. All sera were inactivated by heating for 30 min. at 56°. Phosphate buffered (PB) saline without calcium and magnesium, and 0.2 % (w/v) ethylenediaminetetra-acetic acid in PB saline (EDTA saline) were prepared according to Lwoff, Dulbecco, Vogt & Lwoff (1955).

Disruption of cells. Monkey kidney cells suspended in tissue culture medium in rubber-stoppered cellulose nitrate tubes were disrupted by ultrasonic vibration at 9000 cyc./sec. for 10 min. in a Raytheon 200 W. 10 keye. magnetostrictive oscillator.

Assay of poliovirus infected cells and infectious particles. Poliovirus infected cells and infectious particles were titrated in monkey kidney cell monolayers. The monolayers were washed with serum-free medium and inoculated with 0.5 ml. of a suitable dilution of virus or infected cells mixed with 1 ml. overlay, the final concentration of agar in this thin covering layer being 0.6 %. After the mixture had solidified it was covered with a further 2 ml. overlay and the dishes incubated for 3 days at 37° in air containing 5 % CO_2 . Not less than three samples were assayed from each dilution.

One-step growth curves. For poliovirus in suspensions of monkey kidney cells one-step growth curves were obtained as follows. A monkey kidney cell monolayer was washed twice in PB saline and infected with 0.5 ml. virus suspension, which was allowed to adsorb for 60 min. at 25° or 37°. The monolayer was freed from unadsorbed virus by washing 5 times with PB saline and separated into single cells by incubation with 3 ml. EDTA saline for 30 min. at 37°. The EDTA saline suspension was inactivated with 7 ml. medium 199 and the suspension diluted 1/200 in medium containing 2 % (v/v) inactivated monkey serum. To allow maturation of the virus, 100 ml. of diluted cell suspension were incubated in 250 ml. rubber stoppered siliconed flasks in a 37° water bath and shaken every 30 min. At intervals 0.5 ml. samples were assayed to determine the number of infected cells in the early stages of the experiment, and later the number both of infected cells and of virus progeny spontaneously released into the medium. A second sample (2 ml.) was assayed, after treatment with ultrasonic vibrations to release virus from the cells, to determine the total number of infectious particles. The number of intracellular infective virus was obtained by difference. After dilution the cell suspension contained about 2000 cells/ml., of which 60 were infected, thus virtually precluding early infection of cells by released virus progeny.

Unless otherwise stated, all media and solutions were kept at pH 7.0-7.4 and at 37°.

RESULTS

The same titre of 1.2×10^7 infectious particles/ml. suspension was obtained when the pool of Parker type 1 poliovirus was assayed by the technique described and by the usual method (in which the inoculum is first adsorbed to the monkey kidney cell monolayers, Youngner, 1956), thus showing that all the virus diffused through the semi-solid agar. To ascertain whether the virus matured completely in single cells in suspension in semi-solid agar, a monolayer was inoculated with a dilution of the virus pool estimated to infect about 1000 cells. After it had adsorbed virus the monolayer was separated with EDTA saline and 0.5 ml. samples of the infected cell suspension, suitably diluted, were suspended in 1 ml. semi-solid agar for assay by the present technique. The titre of the virus pool estimated from the number of infected cells so determined was 1.0×10^7 infectious particles. This value, within the limits of experimental error, is the same as the original titre; virus particles adsorbed to cells were capable of completing their replication to the point where the progeny of each cell diffused through the semi-solid agar in a close cluster to form a single plaque in the underlying monolayer.

*The influence of environment on the replication of poliovirus**The effect of temperature*

After adsorption at either 25° or 37° for 1 hr. about 70 % of the adsorbed virus particles were in eclipse. The remainder were released as infectious particles on disintegrating the cells. At 25° the number of infected cells and the proportion of virus which was not in eclipse remained constant for 10 hr. (Table 1), indicating

Table 1. *Effect of incubation at 25° on the survival and replication of poliovirus in suspension of monkey kidney cells at pH 7.0-7.4*

Period after adsorption (hr.)	No. of infected cells (a)	No. of virus particles		% infected cells with infectious virus* (100 b/a)
		In eclipse	Infectious* (b)	
2	75	57	18	24
5	78	48	30	38
5.5	72	54	18	25
6.5	96	81	15	15
7	51	27	24	47
7.5	99	69	30	30
8	63	57	6	10
9	81	51	30	37
10	81	48	33	41

* Infectious particles recovered on disintegrating the cells by ultrasonic vibrations during the eclipse phase.

that the infected cells remained viable and were capable of supporting virus replication on raising the temperature to 37°. By contrast, at 37° the eclipse lasted 3-4 hr., and the 'uneclipsed' particles gradually decreased in number until the reappearance of infective virus at the end of the eclipse period masked the fate of the last few uneclipsed particles (Table 2). Thereafter the intracellular virus increased exponentially until a plateau was reached after 12-14 hr., with a yield of 700-1400 infectious

Table 2. *Poliovirus recovered from infected monkey kidney cells by ultrasonic disruption during the eclipse phase of replication at 37°*

Period after adsorption (hr.)	*No. of infected cells (a)	No. of virus particles		% infected cells with infectious virus (100 b/a)
		In eclipse	Infectious (b)	
2	576	384	192	32
2½	240	216	24	10
3	216	192	24	11
3½	180	171	9	5
4	156	.	453	290†

* Summation of comparable experiments; medium at pH 7.0-7.4.

† Eclipse ended.

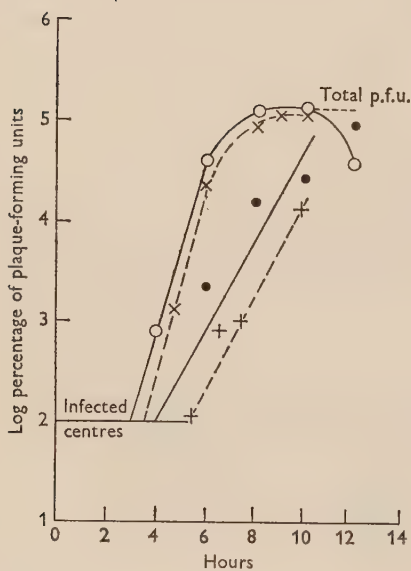


Fig. 1

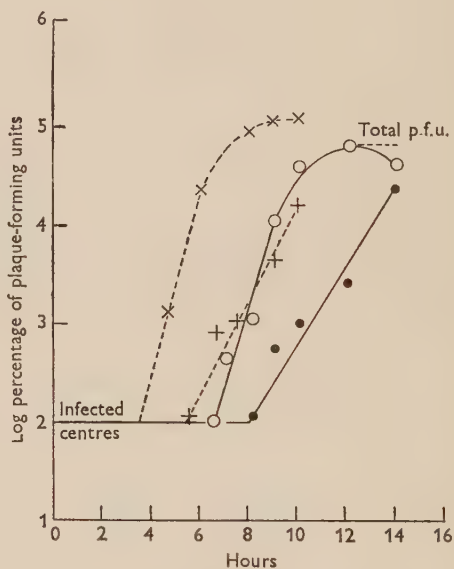


Fig. 2

Fig. 1. The effect of temperature of adsorption on the multiplication of poliovirus. ○—○, intracellular p.f.u.; ●—●, extracellular p.f.u. adsorbed 25°. ×---×, intracellular p.f.u.; +---+, extracellular p.f.u. adsorbed 37°.

Fig. 2. The effect of fluctuation in temperature of incubation on multiplication of poliovirus. ×---×, intracellular p.f.u.; +---+, extracellular p.f.u. incubated 37°. ○—○, intracellular p.f.u.; ●—●, extracellular p.f.u. fluctuated below 37°.

particles from each infected cell. Release began when the number of intracellular infectious particles reached approximately 10 % of the total yield (Figs. 1, 2).

These results suggested that, with the exception of the uneclipsed particles, it would be possible to synchronize replication in all the cells by adsorbing at 25° and then raising the temperature to 37°. However, although the virus particles adsorbed at 25° remained capable of replication, the influence of the initial exposure at 25° on the rate and extent of virus multiplication was unknown, and was accordingly studied. The technique was modified as follows. After adsorption of virus for 1 hr.

at 25°, the monolayer was washed with PB saline at 25° and the temperature raised to 37° by adding EDTA saline at 37° and maintained at that level. There was no significant difference between the multiplication of poliovirus after adsorption at 25° and 37° (Fig. 1).

The slight increase in the duration of the eclipse in cells throughout at 37° is attributable to the method of computation; since virus adsorbed at 37° begins to replicate during the adsorption period, zero time was taken as the time of adding the inoculum to the monolayer although, in fact, replication would not begin until the first particle had been adsorbed and gone into eclipse. At 25° zero time was taken as the moment of raising the temperature to 37°. In this case, we know (Table 1) that replication does not begin until this moment; adsorption at 25° therefore improves the method and has obvious advantages with viruses which are adsorbed slowly. The slight decrease in temperature below 37°, due to removal of the flask containing the virus cell suspension from the water bath to the bench for titration, affected the growth curve by increasing the eclipse period (Fig. 2), and by preventing the eclipse of uneclipsed particles (Table 3).

It is doubtful whether the slight variation in time of release is significant because

Table 3. *Effect of fluctuations in temperature of incubation below 37° on infectious particles recovered by ultrasonic disruption during the eclipse phase of replication*

Period after adsorption (hr.)	*No. of infected cells (a)	No. of virus particles		% Infected cells with infectious virus (100 b/a)
		In eclipse	Infectious (b)	
2	114	81	33	29
4½	117	84	33	28
5	117	99	18	15
5½	108	75	33	30
6	105	72	33	31

Table 4. *Influence of pH value of the medium on the eclipse stage of replication of poliovirus in suspensions of monkey kidney cells*

Period after adsorp- tion (hr.)	pH 5.0-5.5		pH 6.0-6.5		pH 7.0-7.4		pH 8.0+	
	Infected cells*	Intra- cellular infectious particles	Infected cells*	Intra- cellular infectious particles	Infected cells*	Intra- cellular infectious particles	Infected cells*	Intra- cellular infectious particles
2	40	11	.	.	15	4	25	<1
3	10	1	27	<1
3.5	23	2
4	10	31	9	<1
4.5	11	40
5	12	400	13	8
5.5	42	8	25	6
6	42	6	23	40
6.5	46	8	27	140
7	37	10
8	37	<10
9	38	<10

* Mean number of plaques obtained.

release occurred, as it did in other experiments, when 10 % of the total progeny was formed. Moreover, there was no evidence that the lower yield/infected cell after adsorption at 25° was a temperature effect.

The effect of pH

Since the medium employed in these experiments was buffered with sodium bicarbonate, its pH value might have fluctuated during experiments. Replication of poliovirus in cell suspension was therefore studied by the present technique modified in that after adsorption at pH 7.0-7.4 the diluted cell suspension was

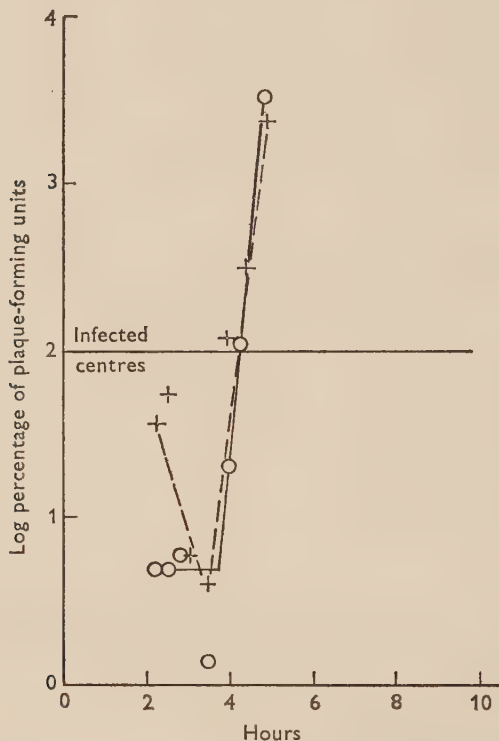


Fig. 3. The effect of antiserum on adsorbed infective particles recoverable by disrupting the cells. O—O, treated antiserum; x---x, control.

prepared in rubber-stoppered flasks with media adjusted to the desired pH value by gassing with CO₂. The pH value was estimated by the colour of the phenol red indicator and by pH papers. At pH 5.0-5.5 poliovirus remained in eclipse for the duration of the experiment (9 hr.; Table 4). At pH 6.0-6.5, the eclipse lasted for 6 hr. and at pH 7.0-7.4 for 3-4 hr. Above pH 8.0 the number of infected cells declined and the number of intracellular particles released by sonic vibrations varied between 0.04 and 3.7/cell, suggesting that in some cases virus had died and in others, that it had managed to replicate to a limited extent. The maintenance of a suitable pH value is, therefore, necessary to obtain reproducible results.

The error introduced by uneclipsed, non-replicating particles

The synchronized replication of poliovirus that follows adsorption at 25° does not of course affect the virus which fails to undergo eclipse. Nevertheless, the later non-synchronous replication of these particles confuses attempts to establish growth rates and virus yields in terms of the controlled conditions. An attempt was therefore made to inactivate these particles by neutralization with immune serum. Their susceptibility to antiserum was investigated as follows. An infected monolayer was dispersed in 3 ml. EDTA saline, and after adding 5 ml. medium 199, the cell suspension was divided into two parts. To 4 ml. was added 1 ml. antiserum and to the other 4 ml., 1 ml. medium as control. After incubation at 37° for 30 min. the suspensions were diluted in the usual manner, thereby rendering the residual antiserum ineffective, and the intracellular virus assayed by the present technique. The uneclipsed infectious particles were immediately decreased to 5 % of the adsorbed virus by treatment with antiserum, whereas those in the control only reached a similar figure by the end of the eclipse phase (Fig. 3). There was no evidence that the remaining uneclipsed particles multiplied. Antiserum therefore inactivated most of the adsorbed virus which did not go into eclipse at 25°, and the technique of estimating synchronized replication was further improved.

DISCUSSION

Youngner (1956) first noted that poliovirus diffused through semi-solid agar overlay to initiate plaque formation in the underlying monkey kidney monolayers. McLaren *et al.* (1959) confirmed this observation but found that only 38 % of the virus formed plaques. They also found, in tests of liquid suspensions of virus, that decreasing the volume of the inoculum increased the efficiency of adsorption. Our results show that all the virus in the overlay formed plaques when the volume of semi-solid agar in which the virus was suspended was decreased to a minimum. Maturation of the virus was also completed in single infected cells dispersed through the overlay, the virus progeny from each cell forming one plaque. Therefore, not only can the actual number of infected cells be estimated directly but the step of adsorbing the virus from liquid medium before covering with overlay is eliminated. This technique was used to study virus multiplication under conditions which virtually preclude multiple infection of the cells. The results emphasize the importance of optimal environmental conditions. One-step growth curves obtained under such conditions of temperature and pH agree with the results of other workers. The eclipse period lasted 3–4 hr.; thereafter intracellular virus progeny increased exponentially until a yield of 700–1400 infectious particles/cell was obtained after 12–14 hr. When the pH value of the medium was suboptimal for the cells, the eclipse was prolonged, a result which is complementary to the finding by Lwoff & Lwoff (1960) that suboptimal pH values decrease the yield of poliovirus in K.B. cells. Suboptimal temperatures prolong the eclipse stage. The completion of virus maturation was not affected, the rate of replication at 37° being the same after adsorption at 25° or 37°. Adsorption at the lower temperature has been used to synchronize the replication of vaccinia virus, which requires a long adsorption period (Furness & Youngner, 1959*a, b*). This method could perhaps also be used to synchronize virus multiplica-

tion in the investigation of interference phenomena, and in genetic recombination where multiple infection of cells with viruses having different adsorption periods might be desired. Low temperatures of incubation have obvious advantages when it is desired to slow or stop the processes of viral synthesis, as for example in the study of biochemical aspects of cell-virus interaction.

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Reduced Oxidative Activities in *Escherichia coli* and *Bacillus megaterium* in Relation to Other Changes during Inhibition of Growth by Streptomycin

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SUMMARY

When the growth of cultures of *Bacillus megaterium* KM, *Escherichia coli* B or *Bacillus subtilis* ceased following addition of streptomycin during the exponential phase of growth, harvested organisms showed oxidative activities on glucose, lactate, pyruvate and succinate which were between 20 and 60 % of those of normal organisms. With *E. coli*, this reduction in oxidative activity was closely parallel to the decrease in growth rate. During the period in which growth became fully inhibited by streptomycin, the total quantities of amino acids and 260 m μ -absorbing compounds in hot water extracts of *E. coli* and *B. megaterium* did not differ significantly from those in normal organisms. Protoplasts could be prepared in normal yield from streptomycin-inhibited organisms of both these species; such protoplasts showed normal stability and had internal osmotic pressures which differed only slightly from normal. From these results it appears unlikely that there was any general breakdown of the permeability barrier of these organisms during inhibition of growth by streptomycin. Lysates of protoplasts from streptomycin-inhibited *Bacillus megaterium* showed oxidative activities which were also decreased to between 20 % and 45 % of those of lysates from normal organisms. With succinate and malate as substrates this difference appeared to be due predominantly to a considerable decrease in the activity of the sedimentable 'ghost' fraction of the lysed protoplasts.

INTRODUCTION

During the period in which growth of cultures of *Staphylococcus aureus* strain Duncan becomes inhibited by streptomycin, there is a progressive decrease in the oxidative activities of harvested organisms which parallels the decrease in the rates of synthesis of cell constituents (Hancock, 1960*a*). In organisms harvested from streptomycin-inhibited cultures, the Q_{O_2} values for oxidation of all substrates are diminished to between 5 and 50 % of the values for normal organisms. Similar observations were made by Jackson (1958) with another strain of *S. aureus*. In *S. aureus* strain Duncan this change was the major alteration observed in biochemical activities of streptomycin-inhibited organisms, and we therefore looked for similar changes in other organisms during inhibition of growth by streptomycin. Attempts have also been made to relate the observed changes in oxidative activity with any general alteration in the permeability of the cells. In *Escherichia coli* strain W, changes occur in the permeability of the cell membrane during inhibition of growth

by streptomycin (Anand & Davis, 1960), and there is evidence that the membrane is the site of an early, but not the bactericidal, action of streptomycin (Drs P. Plotz & B. D. Davis, personal communication). We have also investigated oxidative activities in some subcellular fractions of *Bacillus megaterium* after growth in the presence of streptomycin. Some of these results have been published in abstract form (Hancock, 1960*b*).

METHODS

Organisms, growth and harvesting. *Escherichia coli* strain B was grown in C medium (McQuillen & Roberts, 1954) containing glucose (1 mg./ml.) and adjusted to pH 7.2; *Bacillus megaterium* strain KM and a laboratory strain of *Bacillus subtilis* were grown in nutrient broth (Oxoid no. 2) at pH 7.5. All cultures were grown in flasks shaken at 37° in a constant temperature water bath; up to 100 ml. of medium was used in 250 ml. Erlenmeyer flasks fitted with side arms to fit the Unicam SP. 600 spectrophotometer; larger cultures were grown in 2 l. flasks containing 750 ml. of medium. The pre-warmed growth medium was inoculated with 10 % (v/v) of an overnight culture grown in the same medium in 150 ml. tubes through which a stream of air was bubbled.

Growth was followed by determining the optical density of samples of a culture at 700 m μ by using the Unicam SP. 600 spectrophotometer. When required, streptomycin was added to a portion of a culture during the exponential phase of growth; the remainder of the culture then served as a control.

Organisms were harvested from cultures of small volume (100 ml. or less) on membrane filters as described previously (Hancock, 1960*a*); larger cultures were harvested by centrifugation at 6000 *g* for 5 min. at room temperature. *Escherichia coli* was washed by resuspension in ice-cold 0.5 M-NaCl, since distilled water removes some low-molecular weight compounds from this organism (Britten, 1956). For manometric experiments, *Bacillus megaterium* and *B. subtilis* were washed in distilled water; however, such washing was found to decrease the yield and stability of protoplasts from *B. megaterium* and was omitted when these were to be prepared, since the protoplasts were effectively washed by centrifugation from the 'protoplasting' medium before use.

Manometric techniques. For intact organisms, the conditions were those previously used for *Staphylococcus aureus* (Hancock, 1960*a*). For determining oxidative activity in protoplast lysates and subcellular fractions of *Bacillus megaterium*, 'lysing medium' (see below) was used in manometer flasks in place of the phosphate buffer used for intact organisms. All determinations were carried out in duplicate.

Hot water extracts of organisms were prepared as described previously (Hancock, 1960*a*) after harvesting on membrane filters and resuspension.

Preparation and fractionation of protoplasts. Protoplasts of *Bacillus megaterium* were prepared as described by Weibull, Beckman & Bergström (1959) with sucrose as osmotic stabilizer, but using an initial suspension concentration equivalent to about 15 mg. dry wt. of organisms/ml. When protoplasts were to be prepared simultaneously from normal organisms and streptomycin-inhibited organisms, the volumes of the two suspensions were adjusted initially to give an equal dry weight of organisms/ml. in each. In all subsequent stages the volumes were also adjusted so that the final concentration of protein in the total lysates and other fractions

were similar in the two preparations. Centrifugations were carried out in the No. 40 rotor of the Spinco model L ultra-centrifuge, with refrigeration. Protoplast formation was followed with the phase-contrast microscope, and was complete in 15–20 min. at room temperature. The protoplasts were centrifuged (4000 g, 10 min.), and lysed by suspension in ice-cold 0.04 M-potassium phosphate buffer (pH 7.0) containing 0.002 M-MgSO₄ ('lysing medium'; Storek & Wachsman, 1957); crystalline deoxyribonuclease (L. Light and Co., Ltd., Colnbrook, Bucks) was added to a final concentration of 1 µg./ml. When the viscosity of the lysates had decreased to a normal level, they were used immediately for manometry or further fractionation. The 'ghost' fraction was separated from such lysates by centrifugation at 25,000 g for 15 min., washed once by a similar centrifugation from lysing medium, and resuspended in a volume of cold lysing medium approximately equal to that of the original total lysate.

For manometric experiments particular care was taken that protoplast formation was complete before harvesting and lysis, and that no intact organisms remained in the lysate; on the very infrequent occasions when intact organisms were detected after a thorough microscopic examination, the preparation was discarded. Manometer flasks contained a volume of total lysate containing 5–10 mg. protein, of 'ghost' fraction containing 2–3 mg. protein, or of supernatant fraction containing 5–8 mg. protein.

Osmotically sensitive forms of *Escherichia coli* were formed by the procedure of Mahler & Fraser (1956) using lysozyme and ethylenediaminetetra-acetic acid in 0.5 M-sucrose, and a suspension density of 10–15 mg. dry weight of organisms/ml. Such forms will be referred to here as protoplasts for the sake of convenience only, bearing in mind the reservations of Brenner *et al.* (1958). The development of osmotic sensitivity in suspensions of *E. coli* was followed by pipetting duplicate samples (0.1 ml.) of each suspension into two 2.5 ml. samples of 0.01 M-potassium phosphate buffer (pH 6.8) one of which contained 0.5 M-NaCl; the ratio of the optical density (700 mµ) in buffer to that in buffer + NaCl decreased as fragility developed, and after about 15–20 min. reached a minimum value of 0.18–0.20.

The yield of protoplasts from suspensions of both *Escherichia coli* and *Bacillus megaterium* was estimated by comparing the initial optical density of the suspension with the final optical density when protoplast formation was complete; for this purpose a lower suspension density, 2–3 mg. dry weight of organisms/ml., was used.

Determination of internal osmotic pressure of protoplasts. The osmotic pressure within protoplasts was estimated by a procedure based on method III of Mitchell & Moyle (1956*a*); this depends on determination of the concentration of external solute which is necessary to protect protoplasts against osmotic lysis. Samples (0.1 ml.) of a suspension of protoplasts were pipetted into 2.5 ml. samples of 0.01 M-potassium phosphate buffer (pH 6.8) containing NaCl at concentrations from 0 to 0.5 M. After 20 min., lysis in the more dilute solutions was complete and the optical density (at 700 mµ) of each sample was determined.

Streptomycin. The preparations used in these experiments, and the preparation of solutions, were as described previously (Hancock, 1960*a*).

Amino acids and dry weights of bacterial suspensions were determined as described previously (Hancock, 1960*a*). Substances absorbing light at 260 mµ were estimated by using the Unicam SP. 700 spectrophotometer.

Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951) using suitably diluted samples of the preparations; crystalline lysozyme was used as a standard.

RESULTS

Diminished oxidative activity in streptomycin-inhibited organisms

Streptomycin was used at concentrations which resulted in complete inhibition of growth of the organisms after about 60 min., so that the relative time of occurrence of other changes during this period could be determined. In initial experiments

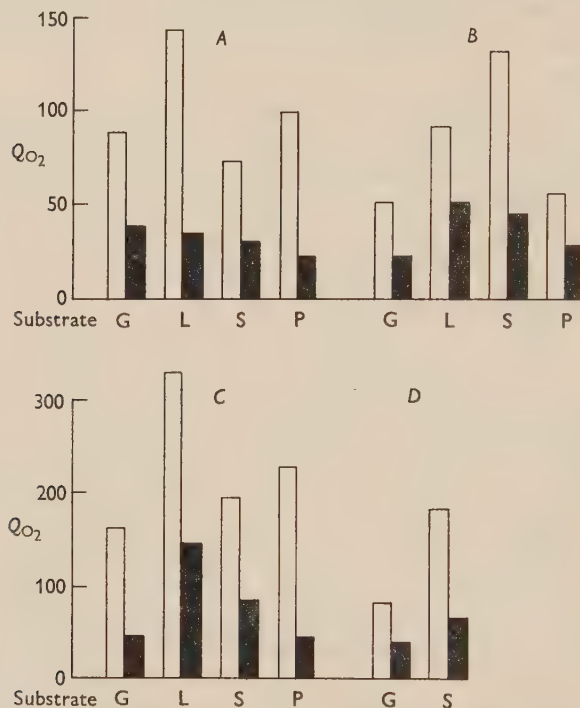


Fig. 1. Q_{O_2} values of organisms harvested simultaneously from control cultures (open columns) and streptomycin-inhibited cultures (solid columns). *A* and *B*. *Bacillus megaterium* KM; streptomycin sulphate 2 μ g./ml. *A*, harvested after 15 min., growth inhibition just detectable; *B*, harvested after 75 min., growth just ceased. (Two separate experiments.) *C*, *Escherichia coli* B, streptomycin sulphate 40 μ g./ml. harvested after 40 min., growth just ceased. *D*, *Bacillus subtilis*, streptomycin sulphate 150 μ g./ml. harvested after 60 min., growth just ceased. Substrates: G = glucose; L = lactate; S = succinate; P = pyruvate.

using *Escherichia coli*, *Bacillus megaterium* and *B. subtilis*, the organisms were harvested immediately after growth had finally ceased in the presence of streptomycin, and their ability to oxidize a number of substrates was compared with that of organisms harvested from a parallel control culture at the same time. The results of these experiments are shown in Fig. 1; Q_{O_2} values were also determined for *Bacillus megaterium* with organisms harvested when the inhibition of growth by streptomycin was first detectable. When inhibition of growth was complete, the

oxidative activities of harvested organisms were decreased in all cases to values considerably below those of normal organisms; with *B. megaterium*, some decrease had already occurred when growth inhibition was first detectable.

To relate these changes more closely to the inhibition of growth, the progress of the decrease of Q_{O_2} (glucose) was compared with the development of growth inhibition in cultures of *Escherichia coli*, with the results shown in Fig. 2. With *E. coli* the Q_{O_2} (glucose) and the growth rate began to decrease at about the same time, and the Q_{O_2} had reached about 30 % of the control value when growth inhibition was complete. These observations are in general very similar to those made with *Staphylococcus aureus* (Hancock, 1960*a*).

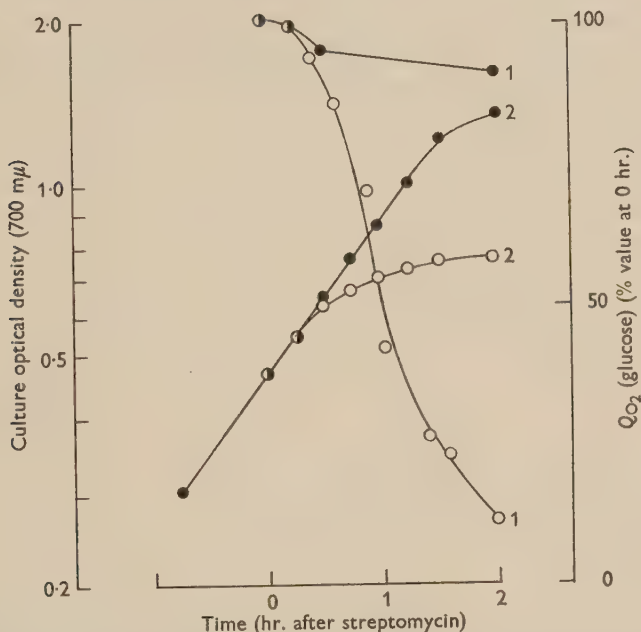


Fig. 2. Q_{O_2} (glucose) values (curves 1) of *Escherichia coli* B harvested from control cultures (●) and cultures after addition of streptomycin sulphate 60 μ g./ml. (○). Curves 2 represent optical densities of the cultures.

Anand & Davis (1960) showed that during the bactericidal action of streptomycin on *Escherichia coli* W, changes occurred in the permeability of the cells which result in the excretion of some intracellular compounds. Changes in permeability could clearly lead to loss of oxidative activity because of loss of respiratory coenzymes, and it was important to determine whether, in the organisms investigated here, any changes in the permeability barrier could be observed during the period in which oxidative activity decreased. No significant decrease in the total quantity of amino acids, 260 $m\mu$ -absorbing substances or phosphate compounds was earlier observed with *Staphylococcus aureus* during inhibition of growth (Hancock, 1960*a*).

Hot-water extractable compounds during inhibition of growth

In the experiment with *Escherichia coli* illustrated in Fig. 2, samples of the suspension of organisms whose Q_{O_2} values were to be determined were taken for estimation of the total quantities of amino acids and 260 $m\mu$ -absorbing compounds in hot-water extracts of the organisms. No large differences were found between the quantities of these compounds in normal organisms and in organisms harvested during the period in which growth became inhibited and in which the Q_{O_2} (glucose) decreased to 30 % or less of the normal value (Table 1). The quantity of amino acids did however begin to decrease after inhibition of growth had become complete. Table 1 includes data obtained in similar experiments with *Bacillus megaterium*. From these results, it appears that the permeability barrier of *E. coli* and *B. megaterium* remained intact, at least to the extent that there was no general leakage of intracellular compounds of low molecular weight from the cells during the period in which growth was inhibited.

Table 1. *Quantities of some compounds in hot-water extracts of Bacillus megaterium KM and Escherichia coli B during inhibition of growth by streptomycin*

The estimations for *E. coli* B were made on the same suspensions as those used in the experiment in Fig. 2. For *B. megaterium*, the streptomycin sulphate concentration was 2 μ g./ml.; inhibition of growth was detectable at 45 min. and complete by 75 min.

C = control organisms; S = streptomycin-inhibited organisms

Organism	Time after streptomycin added (min.)	μ mole amino acids/g. dry weight organisms		Optical density (260 $m\mu$)/g. dry weight organisms	
		C	S	C	S
<i>E. coli</i> B	0	105	105	746	746
	30	—	94	—	1020
	60	96	88	817	900
	90	99	102	746	904
	120	99	51	902	821
<i>B. megaterium</i> KM	0	—	—	1020	1020
	45	111	110	1000	1005
	75	159	145	800	952

Production and stability of protoplasts from streptomycin-inhibited organisms

A second procedure used to investigate the integrity of the permeability barrier depends on comparison of the yield and stability of protoplasts from streptomycin-inhibited organisms and normal organisms. Protoplasts are stable only when suspended in solutions of non-penetrating solutes at concentrations which exert an osmotic pressure on the permeability barrier equal and opposite to that due to the intracellular solutes (for general discussion, see Mitchell & Moyle, 1956*b*). If, in streptomycin-inhibited organisms, alterations were to occur in the permeability barrier which resulted in loss of its ability to exclude the stabilizing solute used (or other molecules such as streptomycin, for example), protoplasts from such organisms would not be stable in the presence of solutes which stabilize protoplasts from normal organisms. In the experiments described here, sucrose or NaCl were used

as stabilizing solutes, and the optical density of a protoplast suspension was used to estimate the proportion of protoplasts which remained intact, as in the experiments of Mitchell & Moyle (1956*a*, 1957). In all cases, streptomycin-inhibited organisms were harvested when inhibition of growth had just become complete; normal organisms were harvested simultaneously from a control culture. Figure 3 shows progress curves of the optical density of suspensions of normal and streptomycin-inhibited *Bacillus megaterium* and *Escherichia coli*, when protoplast formation occurred in the presence of sucrose at a concentration which conferred complete stability on protoplasts from normal organisms, and also when no stabilizer was present. In *B. megaterium* (Fig. 3A) the final yield of protoplasts from streptomycin-inhibited organisms in this experiment was about 20 % lower than that from normal organisms; smaller differences in protoplast yield were obtained in subsequent experiments (Fig. 4A). In *E. coli* (Fig. 3B) there was only a very small difference in the final yield of protoplasts. With both organisms, protoplasts from streptomycin-inhibited organisms were stable for considerable periods of time, so that there appeared to be no leakage of the stabilizing solute into the protoplasts.

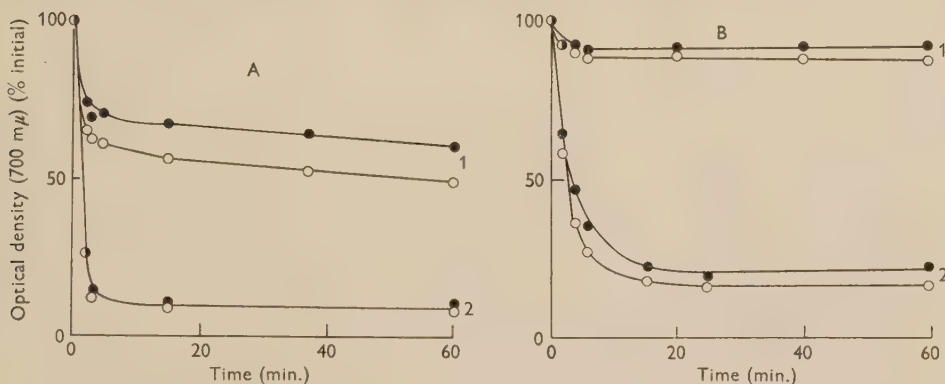


Fig. 3. Optical density of suspensions of *Bacillus megaterium* KM (A) and *Escherichia coli* B (B) during protoplast formation in the presence (curves 1) or absence (curves 2) of 0.5M-sucrose. Bacteria from a culture containing streptomycin sulphate (2 μ g./ml. for *B. megaterium*, 50 μ g./ml. for *E. coli*) were harvested when growth had just ceased (○), and control bacteria were harvested simultaneously (●).

The internal osmotic pressure of protoplasts was estimated by the procedure used by Mitchell & Moyle (1956*a*, 1957) in which the proportion of intact protoplasts is determined over a range of external solute concentrations; the mean internal osmotic pressure is regarded as equal to that of the solution in which 50 % of the protoplasts remain intact. This procedure gives values for the internal osmotic pressure which agree well with those obtained by more direct methods (Mitchell & Moyle, 1956*b*). Figure 4 shows the relation between the concentration of NaCl in the suspending medium and the stability of protoplasts from normal and streptomycin-inhibited *Bacillus megaterium* and *Escherichia coli*. Very similar results were obtained when the stability was measured in solutions containing sucrose in place of NaCl. The yield of protoplasts from the streptomycin-inhibited organisms, expressed relative to that from normal organisms, was 94 % for *B. megaterium* and 97 % for *E. coli*. With *E. coli* protoplasts from streptomycin-inhibited organisms appear to

have about the same mean internal osmotic pressure as those of normal organisms; with *B. megaterium* they had a mean internal osmotic pressure slightly higher than normal. The Q_{O_2} of the organisms used in this experiment were also determined at the same time; with *B. megaterium* the streptomycin-inhibited organisms had Q_{O_2} values between 34 % (succinate) and 50 % (lactate) of the control; with *E. coli* between 28 % (pyruvate) and 49 % (succinate) of the control. From these experiments also it appears that no large leakage of intracellular solutes could have occurred with the streptomycin-inhibited organisms, since such a loss would have resulted in the possession, by protoplasts from these organisms, of a lower mean internal osmotic pressure than protoplasts from normal organisms. It was also intended to investigate the stability and internal osmotic pressure of 'protoplasts' of *Staphylococcus aureus* prepared by the procedure of Mitchell & Moyle (1957);

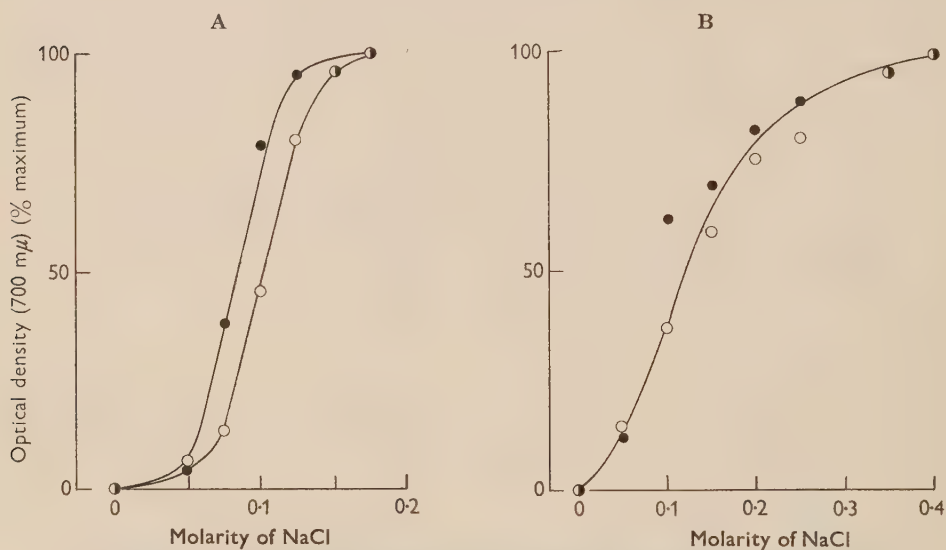


Fig. 4. Final optical densities of suspensions of protoplasts of *Bacillus megaterium* KM (A) and *Escherichia coli* B (B) after transfer to solutions containing NaCl at different molarities, expressed relative to the optical density in 0.5 M-NaCl in which all protoplasts were completely stable. Protoplasts prepared from normal cells (●) and streptomycin-inhibited cells (○), grown as in Fig. 3.

however, for reasons which are not apparent, no 'protoplast' formation could be obtained in suspensions of streptomycin-inhibited organisms, although normal organisms became osmotically fragile.

Oxidative activities in lysed protoplasts of Bacillus megaterium

Attempts were made to investigate oxidative activities in subcellular fractions from streptomycin-inhibited cells of *Bacillus megaterium*. The distribution of a number of oxidative systems in cells of this organism was investigated by Storek & Wachsmann (1957) who used a procedure in which the organisms were first converted to protoplasts, which were then lysed osmotically. The total lysates were further separated into a sedimentable fraction containing 'ghosts' (probably protoplast membranes) and a supernatant fraction. A similar procedure was followed here.

From each suspension of organisms which were to be converted to protoplasts, a sample was first taken to determine the Q_{O_2} values of the intact organisms for subsequent comparison with the activities of subcellular preparations.

Table 2. *Oxidative activities in intact cells and total lysates of Bacillus megaterium KM*

Organisms harvested 60 min. after addition of streptomycin sulphate (1 μ g./ml.) when growth had just ceased in presence of streptomycin

Pre- paration	Substrate	Q_{O_2} (Intact organisms)		μ l. O_2 /mg. protein/hr. (Total lysate)	
		Control	Strepto- mycin- inhibited	Control	Strepto- mycin- inhibited
1	Glucose	187	45.2	13.7	3.5
	Succinate	148	24.9	40.1	11.6
	Lactate	161	18.1	24.1	11.4
2	Succinate	192	32.0	31.4	14.0
	Lactate	148	25.2	61.4	33.6
3	Succinate	242	24.2	46.5	10.8
4	Succinate	225	20.8	32.8	14.6

Table 2 shows the rate of oxidation of some substrates in total lysates of protoplasts from a number of batches of normal and streptomycin-inhibited *Bacillus megaterium*. In each experiment the streptomycin-inhibited organisms were harvested when growth had just ceased, and a control culture was harvested simultaneously. The proportion of the oxidative activity of the intact organisms which was recovered in total lysates ranged between about 3 % for glucose (preparation 1) to 20 % for lactate (preparation 2). The recoveries of activity for succinate and lactate were somewhat higher than those obtained by Storck & Wachsam (1957). In all the preparations, lysates from streptomycin-inhibited *B. megaterium* showed a smaller activity, relative to their protein content, than those from normal organisms. The difference in activity in the total lysates was in most cases somewhat lower than that in the corresponding intact organisms. The addition of streptomycin (up to 100 μ g./ml.) to the contents of the manometer flasks had no effect on the oxidative activities of preparations from normal organisms.

Oxidative activities in fractionated lysates

The systems responsible for the oxidation of succinate and malate were chosen for further investigations; the total activity of these systems is divided about equally between the 'ghost' and supernatant fractions, but the specific activity of the 'ghost' fraction is 3 to 6 times that of the supernatant (Storck & Wachsmann, 1957). It was also found here that activities on these two substrates were more consistent and reproducible than those obtained with other substrates. Although Storck & Wachsmann (1957) found that certain co-factors stimulated oxidative activities in their preparations, the addition of adenosine diphosphate, di- or triphosphopyridine nucleotide, or cytochrome *c*, either singly or together, resulted in no stimulation of the preparations used here.

The distribution of oxidative activities in these fractions from two batches of

normal and streptomycin-inhibited *Bacillus megaterium* are summarized in Table 3. In various preparations, the 'ghost' fraction contained between 15 and 20 % of the protein content of the total lysate, similar to the value found by Storck & Wachsman (1957). These workers observed that the sum of malate oxidizing activity in the two fractions was greater than that in the original total lysate, as was also observed here.

Table 3. *Oxidative activity in fractionated total lysates of Bacillus megaterium KM*

Conditions of growth as in Table 2; C = control organisms,
S = streptomycin-inhibited organisms

Preparation	Substrate	Fraction	Specific activity $\mu\text{l. O}_2/\text{mg. protein/hr.}$		% activity of total lysate	
			C	S	C	S
1	Succinate	Lysate	15.5	9.9	—	—
		Ghosts	35.2	9.6	36.3	15.6
		Supernatant	10.7	10.7	58.1	91.0
2	Succinate	Lysate	17.3	12.7	—	—
		Ghosts	46.5	13.5	45.6	16.1
		Supernatant	9.9	12.8	48.0	85.9
	Malate	Lysate	56.6	31.6	—	—
		Ghosts	235.2	120.0	70.5	57.0
		Supernatant	45.6	39.3	66.7	105.5

With both substrates, there appeared to be no decrease in the oxidative activity in the supernatant fraction from streptomycin-inhibited organism. The decreased activity in the total lysate from such organisms can be ascribed almost entirely to decreased activity in the 'ghost' fraction. When the 'ghost' fraction from normal *B. megaterium* was mixed with supernatant fraction from streptomycin-inhibited organisms, and vice versa, the resulting oxidative activities on succinate were very close to the sum of the activities of each fraction separately (Table 4).

Table 4. *Succinate-oxidizing activity of mixed fractions from normal and streptomycin-inhibited Bacillus megaterium KM*

Conditions of growth as in Table 2; C = control organisms, S = streptomycin-inhibited organisms. Each 'ghost' fraction was resuspended in the same volume as that of the total lysate from which it was prepared. Figures in parentheses are activities to be expected if the activity of each fraction is additive.

		None	Supernatant from	
			C	S
		Activities ($\mu\text{l. O}_2/\text{ml. preparation/hr.}$)		
'Ghosts' from	None	—	71	58
	C	103	190	206
	S	24	(174) 75 (95)	(161) 97 (82)

DISCUSSION

The results described here would appear to exclude the occurrence of any general breakdown of the permeability barrier of *Bacillus megaterium* KM or *Escherichia coli* B during the period in which inhibition of growth by streptomycin becomes complete. With *E. coli* there were no large changes in the total intracellular pools of amino acids or of 260 m μ -absorbing compounds during this period. Moreover, protoplasts were prepared in normal yield from both these organisms when the inhibition of growth was complete; such protoplasts had a stability and a mean internal osmotic pressure which differed only slightly if at all from those of protoplasts from normal organisms. Even when streptomycin-inhibited organisms showed Q_{O_2} values which were 30 % or less of those of control organisms, they could be converted to stable protoplasts, and were indeed used routinely to prepare protoplasts and total lysates. These results do not, however, exclude the possibility of changes in permeability to specific intracellular compounds or groups of compounds, such as the excretion of certain mononucleotides in *E. coli* W (Anand & Davis, 1960), or of general changes in permeability too small to observe by the methods used here, but which might be detectable by investigating compounds released into the growth medium as was done by Anand & Davis (1960).

A decrease in oxidative activities accompanies the inhibition of growth by streptomycin in *Escherichia coli* B and *Bacillus megaterium* KM, as well as in two strains of *Staphylococcus aureus* (Jackson, 1958; Hancock, 1960*a*) and in *Bacillus pumilus* (J. W. Lightbown, personal communication). With the techniques used here, conclusions as to whether this decrease precedes or follows the decrease in growth rate should be made with reservations, but the two changes are certainly closely parallel; more precise investigation of the relationship might perhaps be obtained by continuously recording the oxygen consumption of a culture. There would appear to be no *a priori* reason for a decreased oxidative activity which occurs as a non-specific accompaniment of a decreased growth rate; no such change occurred in *S. aureus* after inhibition of growth by penicillin (Gale & Taylor, 1947) or other antibiotics (Hancock, 1960*a*).

Since protoplast lysates from streptomycin-inhibited *Bacillus megaterium* showed decreased oxidative activities relative to preparations from normal organisms, it would not appear that permeability differences are responsible for the difference in activity of the corresponding intact organisms. As differences in activity were found even in the separated 'ghost' fraction, it seems unlikely that the absence of diffusible cofactors, or the presence of diffusible inhibitors, is responsible for the decreased activity of lysates from streptomycin-inhibited organisms; the observed additive activities of mixed 'ghosts' and supernatant fractions (Table 4) would also support this conclusion. With the systems which oxidize succinate and malate, the decreased activity of total lysates appears to be due predominantly to decreased activity of the 'ghost' fraction; the activity of the supernatant fraction was not affected. The succinate-oxidizing activity in the 'ghost' fraction also differs from that in the supernatant fraction in that it is stimulated about ten-fold by phenazine methosulphate under the conditions used (Kearney & Singer, 1956) for assaying succinic dehydrogenase, whereas the activity in the supernatant fraction shows no stimulation. It is known that the 'ghost' fraction contains virtually all of the cytochrome system

of *B. megaterium* KM (Weibull, 1953). Decreased activity of multi-enzyme systems, such as those which oxidize succinate and malate, could clearly be caused either by alterations in the activity of enzymes *per se*, or by structural changes in the 'ghosts'. With *B. megaterium* there is as yet no other evidence for such a structural change, and no apparent differences can be seen by phase-contrast microscopy between the ghosts from normal and streptomycin-inhibited organisms.

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Ability of *S*-Methyl-L-cysteine to Annul the Inhibition of Yeast Growth by L-Ethionine and by *S*-Ethyl-L-cysteine

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SUMMARY

The inhibition of growth of a brewer's yeast and four other yeasts by L-ethionine, and the annulment of the inhibition by L-methionine were examined. *S*-Methyl-L-cysteine, DL-methionine sulfoxide and DL- α -amino-*n*-butyric acid were also able to decrease the growth inhibition. At a fivefold molar concentration, *S*-methyl-L-cysteine annulled completely the effect of ethionine on the brewer's yeast. *S*-Ethyl-L-cysteine was ineffective and in certain conditions it acted as a growth inhibitor; this inhibition was annulled by methionine and by *S*-methylcysteine.

INTRODUCTION

The inhibitory effect of ethionine on microbial growth was first observed by Harris & Kohn (1941) with *Escherichia coli*. These workers also recognized the ability of methionine to abolish the growth inhibition, thus supporting the original suggestion of Dyer (1938) that ethionine is a metabolic antagonist of methionine. Ethionine inhibition of growth in *E. coli* was also reported by Roblin *et al.* (1945) and a similar effect with baker's yeast was obtained by Loveless, Spoerl & Weisman (1954). The antagonistic effect of ethionine on methionine utilization has also been shown with *Lactobacillus arabinosus* (Camien & Dunn, 1950) and *L. mesenteroides* (Kihara & Snell, 1955). It has been found that ethionine is not metabolically inert, but undergoes a number of reactions analogous to those characteristic of methionine. In *Torula utilis* and baker's yeast it is converted to 5'-ethylthioadenosine (Schlenk & Tillotson, 1954*a*) and *S*-adenosylethionine (Parks, 1958), the latter compound being capable of undergoing transethylation in yeast. The intact molecule of ethionine has been shown to enter into the cell protein of *Tetrahymena pyriformis* (Gross & Tarver, 1955) and into the α -amylase of *Bacillus subtilis* (Yoshida, 1958; Yoshida & Yamasaki, 1959). Ethionine may therefore inhibit growth through a number of mechanisms, for example, by competition with methionine, thereby blocking essential transmethylation systems, by undergoing transethylation reactions resulting in the formation and accumulation of ethyl analogues of important methylated metabolites, and by participating in protein synthesis with the formation of abnormal proteins containing ethionine in place of methionine. The present paper describes a further examination of the ethionine inhibition of yeast growth, in which a number of compounds related structurally to methionine have been tested for their ability to annul the inhibition. One compound which is effective in

this respect is *S*-methyl-L-cysteine. It was also found that under certain conditions *S*-ethyl-L-cysteine exhibits growth-inhibitory properties which, like those of ethionine, are annulled by methionine and by *S*-methylcysteine.

METHODS

Yeasts. The brewer's yeast used was a Guinness strain of *Saccharomyces cerevisiae* as in previous studies (Maw, 1960, 1961). The other yeasts used were a baker's yeast, a strain of *S. carlsbergensis* (NCTC 7014), a strain of *Torula utilis* (NCTC 3576) and a strain of *Candida lipolytica* (Harrison) Diddens & Lodder.

Compounds used. *S*-Methyl-L-cysteine was prepared by the reduction and subsequent methylation of L-cystine in anhydrous ammonia (du Vigneaud, Loring & Craft, 1934). The other compounds used were commercial samples, obtained from British Drug Houses Ltd., the Nutritional Biochemicals Corporation, or the California Foundation for Biochemical Research. The following amino acids were obtained in the form of their L-isomers: methionine, ethionine, *S*-methylcysteine, *S*-ethylcysteine, cysteine, glutamic acid and aspartic acid. The remaining amino acids were available only in their DL-forms.

Media. The growth medium used for the brewer's yeast was similar to that described previously (Maw, 1960) but with the following additions (the amounts given are for 1 l. final medium): $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 180 $\mu\text{g.}$; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 170 $\mu\text{g.}$; H_3BO_3 , 300 $\mu\text{g.}$; $(\text{CH}_3\text{CO}_2)_2\text{Zn} \cdot 2\text{H}_2\text{O}$, 3.4 mg. Sulphate was added to a final concentration of 0.312 mM (10 mg. sulphate-S/l.). This medium is hereafter referred to as the sulphate medium. The yeast was also made sulphur-deficient by growing it in a sulphate-free medium. This corresponded in composition to the sulphate medium except that sodium sulphate was omitted. The other yeasts were grown on the sulphate medium further supplemented with *p*-aminobenzoic acid (100 $\mu\text{g./l.}$) and nicotinic acid (100 $\mu\text{g./l.}$).

Growth experiments. Yeasts were grown for 2 days at 30°. The organisms were then centrifuged off, resuspended three times in sterile saline and recentrifuged, and then suspensions prepared in saline to contain about 0.3 mg. dry weight yeast/ml. Growth experiments were carried out in 25 ml. conical flasks containing 10 ml. medium and other reagents to which was added 0.5 ml. yeast suspension. Flasks were shaken at 30° in a thermostat and the amount of growth determined turbidimetrically after 40 hr. (Maw, 1960).

RESULTS

Experiments with L-ethionine

Effect of L-ethionine on yeast growth. The effect of various concentrations of L-ethionine on the growth of the brewer's yeast in a medium containing 0.312 mM-sulphate was examined. When the yeast had been grown previously on the sulphate medium, 0.312 mM-ethionine produced a growth inhibition of 90–100 %, whereas growth inhibition was small with concentrations below 10 μM (see Fig. 1*a*).

The yeast was made sulphur deficient by growing it for several weeks in a sulphate-free medium. When it was then tested for growth in the sulphate medium in the presence of ethionine it was found to be markedly more sensitive to the inhibitor. Complete growth inhibition was obtained with ethionine at concentrations down to 3.12 μM ; at 0.78 μM a 69 % inhibition was observed (see Fig. 1*b*).

In the case of the four other yeasts studied, the growth of *Candida lipolytica* was partially suppressed (31 %) by $31.2 \mu\text{M}$ ethionine, that of the baker's yeast, *Saccharomyces carlsbergensis* and *Torula utilis* being unaffected. At 0.312 mM ethionine produced complete growth inhibition of *C. lipolytica*, *S. carlsbergensis* and *T. utilis*, but only partial growth inhibition (67 %) of baker's yeast. Increasing the ethionine to 1.56 mM only increased the growth inhibition of baker's yeast to 83 %.

Effect of L-methionine on the inhibition of growth caused by ethionine. Equimolar amounts of L-methionine completely annulled growth inhibition in the normal brewer's yeast at all concentrations of L-ethionine used ($31.2 \mu\text{M}$ to 1.56 mM). Lower

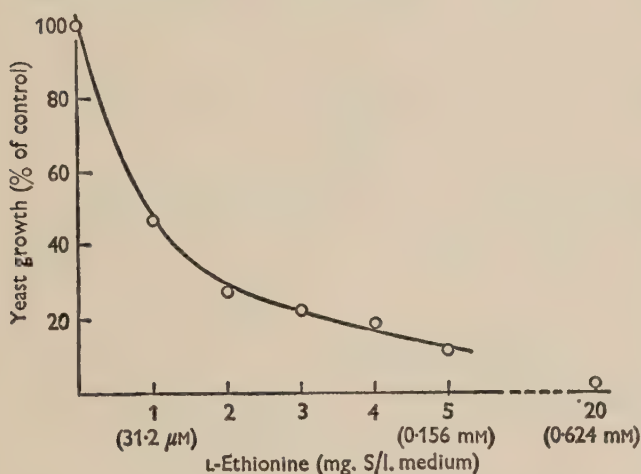


Fig. 1a

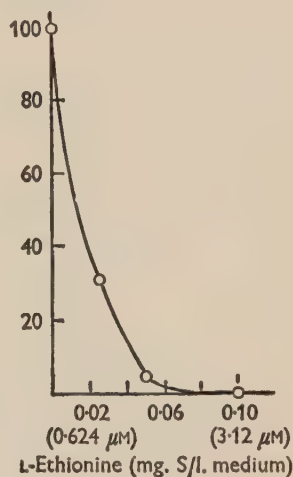


Fig. 1b

Fig. 1a. Effect of L-ethionine on the growth of normal brewer's yeast.

Fig. 1b. Effect of L-ethionine on the growth of sulphur-deficient brewer's yeast.

concentrations of methionine produced only partial decrease of the inhibition. This is illustrated in Fig. 2a for ethionine at 0.312 mM . Essentially similar results were obtained when methionine was present in the medium as the sole source of sulphur for growth. Methionine was also equally effective in annulling the inhibition in the sulphur-deficient brewer's yeast grown on the sulphate medium. Methionine $31.2 \mu\text{M}$ promoted full growth in the presence of the same concentration of ethionine, which alone produced complete growth inhibition. Growth inhibition by ethionine of the four other yeasts under study was also completely abolished by equimolar amounts of methionine.

Effect on ethionine inhibition of compounds related to methionine. The specificity of methionine in annulling ethionine inhibition of growth was studied with the brewer's yeast. The compounds examined included a number with close structural relationships to methionine and others which are known to be related metabolically to this amino acid. L-Ethionine was used at 0.312 mM and 1.56 mM , and the various compounds under test were added at these concentrations and at five times these values. The findings obtained with the two ethionine concentrations were closely similar. Table 1 gives the summarized data obtained with 0.312 mM ethionine. It will be seen that in addition to methionine itself, only three compounds, namely

DL-methionine sulphoxide, DL- α -aminobutyric acid and S-methyl-L-cysteine possessed any marked ability to annul the inhibition. All three exerted an effect at five times the ethionine concentration, but at equimolar concentrations methionine sulphoxide was ineffective while the other two compounds were only slightly

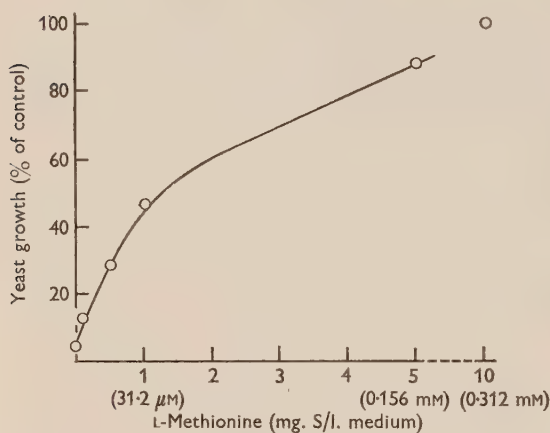


Fig. 2a

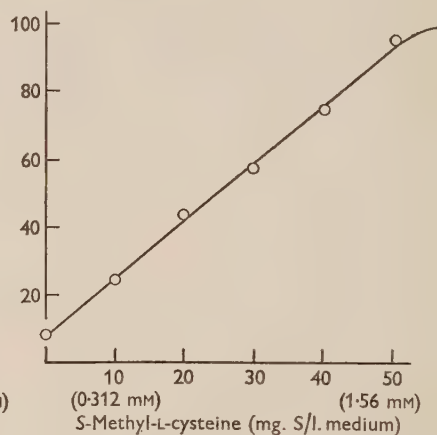


Fig. 2b

Fig. 2a. Annulment by L-methionine of the growth-inhibitory effect of L-ethionine (0.312 mm) on brewer's yeast.

Fig. 2b. Annulment by S-methyl-L-cysteine of the growth-inhibitory effect of L-ethionine (0.312 mm) on brewer's yeast.

Table 1. *Effect of various compounds related to methionine on the inhibition of growth of brewer's yeast by L-ethionine*

L-Ethionine in medium at 0.312 mm (10 mg. ethionine-S/l.). Added compounds at 0.312 mm and 1.56 mm. Growth of yeast determined in flasks shaken for 40 hr. at 30° and expressed as % of the growth obtained in the sulphate medium in the absence of ethionine. Growth in the presence of L-ethionine (0.312 mm) alone 5.4 %.

Added compound	Growth of yeast when added compound was	
	0.312 mm	1.56 mm
	Relative growth (%)	
S-Methyl-DL-methionine chloride	11.8	12.1
DL-Methionine sulphoxide	4.0	99.1
DL-Methionine sulphone	1.0	16.5
α -Ketomethionine	4.6	24.1
DL-Homocysteine	6.0	13.1
DL- α -Amino-n-butyric acid	18.3	51.5
DL-Homoserine	8.0	16.4
L-Glutamic acid	11.0	17.7
L-Aspartic acid	9.9	15.0
L-Cysteine	7.3	5.1
Glutathione (reduced)	2.5	14.4
S-Methyl-L-cysteine	21.5	94.0
S-Ethyl-L-cysteine	5.9	10.0
Sodium methylthioacetate	5.9	5.9
S-Methyl-L-cysteine (0.312 mm) + DL- α -amino-n-butyric acid (0.312 mm)	31.4	—

effective. Other compounds tested included adenine, adenosine and *p*-amino-benzoic acid. None of these had any effect on the inhibition.

Effect of S-methyl-L-cysteine on the growth inhibition by ethionine. The ability of *S*-methyl-L-cysteine to antagonize the growth inhibition due to L-ethionine is further illustrated in Fig. 2*b*. (The medium contained sulphate as the sulphur source together with 0.312 mM-ethionine). For ethionine concentrations within the range 31.2 μ M to 1.56 mM equimolar amounts of *S*-methylcysteine generally decreased the growth inhibition by 20–30 %, but a fivefold concentration restored growth completely or almost so.

S-Methylcysteine also decreased the growth inhibition of other yeasts, although its effect was somewhat less marked than with the brewer's yeast (see Table 2).

Table 2. *Effect of L-methionine and of S-methyl-L-cysteine in annulling the growth inhibition due to L-ethionine in various yeasts*

L-Ethionine in medium 0.312 mM. Growth of yeasts determined in flasks shaken for 40 hr. at 30° and expressed as % of the growth obtained in the sulphate medium in the absence of ethionine.

Yeast	% Inhibition of growth in the presence of			
	Ethionine alone (0.312 mM)	Ethionine (0.312 mM) + methionine (0.312 mM)	Ethionine (0.312 mM) + <i>S</i> -methylcysteine	
			(0.312 mM)	(1.56 mM)
Baker's yeast	67.3	0	46.3	0
<i>S. carlsbergensis</i>	100	12.5	100	52.9
<i>C. lipolytica</i>	97.7	3.5	70.5	36.2
<i>T. utilis</i>	96.5	3.5	100	50.8

Experiments with S-ethyl-L-cysteine

S-Ethyl-L-cysteine and yeast growth. In view of the relationships between ethionine, methionine and *S*-methylcysteine in connexion with yeast growth, it seemed of interest to examine the related compound *S*-ethyl-L-cysteine to determine whether it paralleled *S*-methylcysteine in protecting against ethionine inhibition or whether it resembled ethionine in being itself growth inhibitory. At concentrations up to 3.12 mM in an otherwise sulphur-deficient medium *S*-ethylcysteine was completely unable to act as a sulphur source, in contrast to *S*-methylcysteine (Maw, 1960, 1961). Furthermore, unlike *S*-methylcysteine, the ethyl compound showed no detectable ability to annul growth inhibition by 0.312 mM-ethionine, even when present at ten times this concentration (see also Table 1).

Growth of the brewer's yeast and the four other yeasts studied in the sulphate medium was also not suppressed by *S*-ethylcysteine itself at concentrations up to 3.12 mM. However, the compound showed distinct growth-inhibitory properties towards the brewer's yeast (*a*) when the yeast was first made sulphur-deficient before being tested for growth in the sulphate medium, or (*b*) when it was grown in a medium containing *S*-methylcysteine as the sole sulphur source.

Effect of S-ethyl-L-cysteine on the growth of sulphur-deficient brewer's yeast. When made sulphur deficient the brewer's yeast proved to be particularly sensitive to the presence of *S*-ethyl-L-cysteine (see Fig. 3). *S*-Ethylcysteine was completely growth

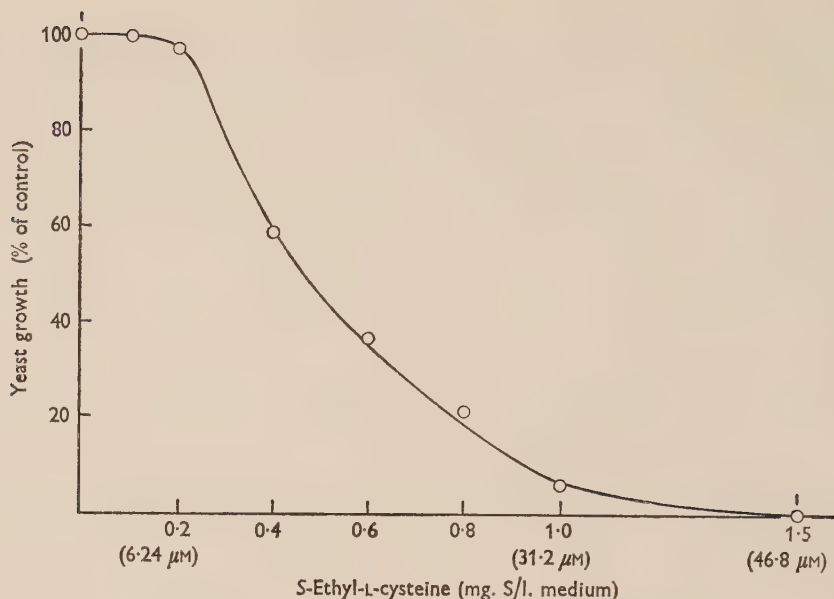


Fig. 3. Effect of *S*-ethyl-L-cysteine on the growth of sulphur-deficient brewer's yeast.

inhibitory at concentrations down to $31.2 \mu\text{M}$. This growth inhibition, like that produced by ethionine, was annulled by both L-methionine and *S*-methyl-L-cysteine (see Fig. 4*a* and 4*b*; the *S*-ethylcysteine concentration in these experiments was 0.312 mM). Methionine was the more active compound in annulling the inhibition. For concentrations of *S*-ethylcysteine in the medium within the range 0.312 mM to 3.12 mM , methionine at one-fourth of the concentration produced complete

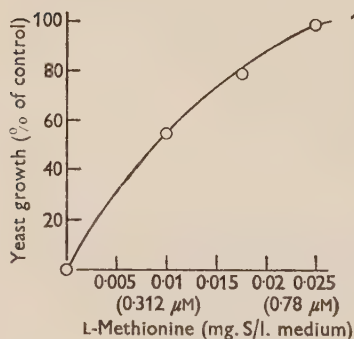


Fig. 4*a*

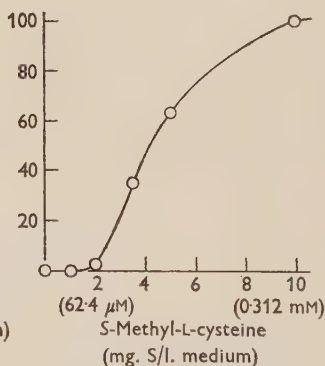


Fig. 4*b*

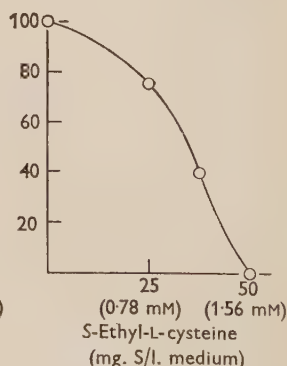


Fig. 5

Fig. 4*a*. Annulment by L-methionine of the growth-inhibitory effect of *S*-ethyl-L-cysteine (0.312 mM) on sulphur-deficient brewer's yeast.

Fig. 4*b*. Annulment by *S*-methyl-L-cysteine of the growth-inhibitory effect of *S*-ethyl-L-cysteine (0.312 mM) on sulphur-deficient brewer's yeast.

Fig. 5. Effect of *S*-ethyl-L-cysteine on the growth of brewer's yeast in the presence of *S*-methyl-L-cysteine (0.312 mM) as sulphur source.

restoration of growth, and even at one-thousandth of the concentration restored 50 % growth. *S*-Methylcysteine gave complete restoration of growth when present in equimolar concentrations, but at lower concentrations it caused only partial annulment of the inhibition.

Growth inhibition of brewer's yeast with S-methyl-L-cysteine as sulphur source. Although the brewer's yeast grown in the presence of sulphate was unaffected by *S*-ethyl-L-cysteine even in relatively high concentrations, growth inhibition was observed when *S*-methyl-L-cysteine was made the sole sulphur source. Figure 5 shows the inhibitory effect of the ethyl compound in the presence of 0.312 mM-*S*-methylcysteine. In contrast, when L-methionine (0.312 mM–1.56 mM) was used as the sulphur source, *S*-ethylcysteine showed no growth-inhibitory properties.

DISCUSSION

The evidence so far available points to the biological role of ethionine as a competitive antagonist of methionine in reactions subsequent to the synthesis of the latter, e.g. conversion to *S*-adenosylmethionine (Parks, 1958) and incorporation into protein (Gross & Tarver, 1955). The present work indicates that only a very limited number of compounds acted like methionine in annulling growth inhibition by ethionine. The effectiveness of methionine sulphoxide in this respect seems most probably to be accounted for by the recent demonstration by Black *et al.* (1960) of an enzyme system in yeast which catalyses the reduction of the sulphoxide to methionine. A somewhat unexpected finding was the inability of *S*-methylmethionine to annul ethionine inhibition, in view of the existence in yeast of a trans-methylase capable of demethylating *S*-methylmethionine to methionine (Shapiro, 1958). Any explanation of this, however, would need to take into account the extent to which *S*-methylmethionine can penetrate the intact yeast cell, about which little is known.

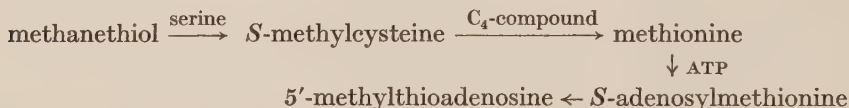
The ability of *S*-methylcysteine to annul the growth inhibition points to a close metabolic link between this amino acid and methionine. *S*-Methylcysteine and its sulphoxide occur in the non-protein nitrogen fraction of various plants (Morris & Thompson, 1955; Synge & Wood, 1956; Thompson, Morris & Zacharius, 1956), and there is evidence for its presence in the mycelium of *Neurospora crassa* (Ragland & Liverman, 1956). Furthermore, Wolff, Black & Downey (1956) demonstrated its enzymic synthesis in baker's yeast from methanethiol and serine. *S*-methylcysteine is able to act as a source of sulphur for the growth of certain yeasts (Margolis & Block, 1958; Maw, 1960, 1961), *Escherichia coli* (Roberts *et al.* 1955) and certain strains of *N. crassa* (Ragland & Liverman, 1956). In brewer's yeast, when present in sufficient amounts, it can promote optimum growth. The sulphur of *S*-methylcysteine is therefore available for the synthesis of methionine and other sulphur amino acids. However, *S*-methylcysteine may be distinguished from other compounds capable of acting as sulphur sources, e.g. cysteine and glutathione, in that such compounds are ineffective in annulling the ethionine inhibition.

Three possible explanations to account for the antagonism between *S*-methylcysteine and ethionine have been considered: (i) *S*-methylcysteine may substitute for methionine in one or more reactions essential for growth which have been blocked by ethionine; (ii) *S*-methylcysteine may be a normal intermediate in certain

reactions of methionine; (iii) in addition to blocking the further metabolism of methionine, ethionine may act as an inhibitor at some stage in methionine *synthesis* from sulphate and cysteine, and *S*-methylcysteine may abolish the ethionine effect either (a) by entering the pathway of methionine synthesis above this step, or (b) by giving rise to methionine by a separate pathway unaffected by ethionine.

There is no clear-cut evidence to support (i) or (ii). The apparent absence of *S*-methylcysteine from proteins argues against (i). Furthermore, if (ii) were correct, *S*-methylcysteine would need to be an intermediate in the major metabolic reactions of methionine, such as protein synthesis and *S*-adenosylmethionine formation, for the compound to be able to bypass completely the growth-inhibitory effects of ethionine.

The most tenable explanation seems to be (iii). Since cysteine is unable to annul ethionine inhibition, it is unlikely that *S*-methylcysteine gives rise to methionine through the intermediate formation of cysteine, and this would at the same time imply that ethionine inhibits at a stage in the synthesis after cysteine. Conversion of *S*-methylcysteine to cysteine also seems unlikely from the observation of Ragland & Liverman (1956) that the methyl compound acts as a sulphur source for strains of *Neurospora crassa* able to grow on methionine but unable to utilize cysteine. The idea of a separate pathway from *S*-methylcysteine to methionine, not involving cysteine, is suggested by the work of Wolff *et al.* (1956) and Schlenk & Tillotson (1954*b*) on the conversion in yeast of methanethiol to *S*-methylcysteine and 5'-methylthioadenosine. This is believed to proceed by the following scheme:



The identity of the intermediates between *S*-methylcysteine and methionine is not known, but the step is thought to be a transthio-methylation to a C_4 -compound. In this connexion, it was found in the present work that an equimolar mixture of *S*-methylcysteine and α -aminobutyric acid was unable to act in place of an equivalent amount of methionine in annulling ethionine inhibition (see Table 1).

The annulment of the growth-inhibitory effect of *S*-ethylcysteine by methionine and by *S*-methylcysteine further illustrates the metabolic link between these last two compounds. *S*-Ethylcysteine was shown to be a tuberculostatic agent by Brown *et al.* (1954) and to inhibit penicillin synthesis in *Penicillium chrysogenum* by Demain (1956). This compound would be expected to block the incorporation and/or metabolism of *S*-methylcysteine. The annulment by extremely low concentrations of methionine of the growth inhibition due to *S*-ethylcysteine suggests a further site of action for the compound, possibly through the formation of ethionine or a related compound.

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The Action of Phospholipase A and Lipid Solvents on Murray Valley Encephalitis Virus

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SUMMARY

Phospholipase A (100 $\mu\text{g./ml.}$) acting at 37° for 30 min. decreased the infective titre of crude or purified Murray Valley encephalitis (MVE) virus from 7 or 8 log to less than 1 log. This inactivated virus yielded 2 log of infective 'ribonucleic acid' ('RNA') when it was treated with phenol. Ether, chloroform or butanol acting at 0-4° each decreased the infectivity of crude or purified MVE virus. Again the inactivated virus yielded 'RNA' with an infective titre higher than the titre of the inactivated virus. Infective 'RNA' prepared from crude MVE virus was not inactivated by ether, chloroform or butanol. High concentrations of phospholipase A preparations destroyed 'RNA', possibly due to traces of contaminating ribonuclease. The results suggest that intact viral phospholipid is necessary for infectivity of the virus particle.

INTRODUCTION

That arthropod-borne viruses contained lipid was first clearly shown by Taylor, Sharp, Beard & Beard (1943) from a chemical analysis of eastern equine encephalomyelitis virus. Birch (1941) demonstrated the inactivation of an arthropod-borne virus (western equine encephalitis) by diethyl ether, and Hammon, Reeves & Izumi (1942) described the inactivation of St Louis encephalitis virus by diethyl ether. Andrewes & Horstmann (1949) introduced this reaction to assist in grouping viruses, and showed that ether decreased the titre of yellow fever virus by 3 log, and of louping ill by 2 log. Sunaga, Taylor & Henderson (1960) found that ten arthropod-borne viruses were decreased in titre by treatment with diethyl ether; for example a preparation of Japanese B encephalitis virus was decreased in titre from 3.5 log to less than 1.0 log. The present paper examines the action of phospholipase A, ether, chloroform and *n*-butanol on the infectivity of crude and purified preparations of the virus of Murray Valley encephalitis (MVE virus) and on the infective 'RNA' of this virus. Some of the present results with phospholipase A have already been published in brief (Anderson & Ada, 1960).

METHODS

Murray Valley encephalitis (MVE) virus. Crude virus was a 20 % (w/v) suspension of infected suckling mouse brain in phosphate buffered saline (pH 7.7). Purified virus was prepared by treatment with protamine, ultracentrifugation, adsorption to and elution from hydroxyl apatite and a second ultracentrifugation (Ada, Anderson & Abbot, 1961). The purified virus (deposit from second centrifugation) was suspended in a solution containing NaHCO_3 (0.025 M), cystine (0.85 M), NaCl (0.15 M) and buffered with phosphate (0.25 M; pH 7.7).

'RNA' was prepared by phenol treatment of crude MVE virus (Anderson & Ada, 1959a). This standard infective 'RNA' should be distinguished from the infective 'RNA' prepared by a similar phenol treatment of certain preparations of damaged virus mentioned in the text.

Treatment of virus with deoxycholate. Sodium deoxycholate was added to virus suspensions to a final concentration of 1 % (w/v). The mixture was held at room temperature for ten minutes (Anderson & Ada, 1959b).

Phospholipase A. This was isolated from the venom of the Black Snake (*Pseudechis porphyriacus*) according to Doery & Pearson (1961). Before use the enzyme preparation was diluted in physiological saline (pH 6.0) to a concentration of 100 $\mu\text{g./ml.}$ and placed in a boiling water bath for 10 min.

Ribonuclease (Worthington or Delta) was diluted in physiological saline before use.

Lysolecithins. These were prepared by the action of phospholipase A on egg lecithin and L- α -(dimyristoyl) lecithin (Doery & North, 1960) and supplied to us by Miss H. Doery.

n-Butanol. Laboratory grade reagent was redistilled and a fraction with a boiling point range 116°–118° was used.

Chloroform. Anaesthetic grade was used.

Ether. Diethyl ether was prepared for use as described previously (Anderson & Ada, 1959a).

Phosphate buffered (PB) saline. An aqueous solution containing NaH_2PO_4 (0.05 M), NaOH (0.044 M) and NaCl (0.13 M), (pH 7.7) was used.

Normal baby mouse brain extract (BMBE). This was a 20 % (w/v) emulsion in PB saline of brains of mice 8–10 days old. The product was centrifuged (2000 g, 5 min., 0–4°) and used fresh.

Normal rabbit serum. Rabbits were bled from the ear vein and the serum separated at 37°. Pooled serum was filtered through a Seitz filter pad and stored at –20°. As diluent for virus it was used as a 10 % dilution in physiological saline.

Treatment of virus or 'RNA' with lipid solvent. Two volumes of solvent were added to one volume of virus or 'RNA' contained in a glass-stoppered bottle big enough for the bottle to be less than half full. The mixture was shaken (100 strokes/min.; amplitude 5 cm.) at 0° for the appropriate time.

Titration of infectivity for virus and 'RNA'. Titration in 12-day chick embryos was done on the chorioallantoic membrane, the membranes being 'dropped' with the inoculum of 0.05 ml. The infectivity was determined as the 50 % lethal end-point at 3 days.

RESULTS

Action of phospholipase A on MVE virus

Crude MVE virus was mixed with phospholipase A to a final concentration of 100 $\mu\text{g.}$ enzyme/ml., and the mixture held at 37° for 30 min. The infective titre decreased from 7.0 to 0.4 log, while virus in a control preparation did not decrease in titre. Both the control and the enzyme-treated virus were then treated with phenol by the standard method for preparation of infective 'RNA'. Control virus yielded 4.5 log of infective 'RNA', enzyme-treated virus yielded 2.5 log of 'RNA'. Purified MVE virus gave essentially similar results (Table 1).

Table 1. *Action of phospholipase A (100 $\mu\text{g.}$ /ml.) on MVE virus (37°, 30 min.)*

	Control virus*	Virus treated with phospholipase A
Crude virus		
Titre of virus	7.0†	0.4
Titre of 'RNA'‡	4.5	2.5
Purified virus		
Titre of virus	8.0	0.6
Titre of 'RNA'	5.0	1.6

* Control virus held at 37° for 30 min. in absence of phospholipase A.

† \log_{10} infectivity titre.

‡ 'RNA' was prepared by the action of phenol on virus which had been held at 37° for 30 min., either with or without phospholipase A.

The activity of phospholipase A against virus was titrated in the above system. Serial 10-fold dilutions of enzyme were mixed with equal volumes of virus and incubated at 37° for 30 min.; control virus was incubated without enzyme. The endpoint was taken as the concentration of enzyme which destroyed 2 log of infectivity. The titre against either undiluted crude virus or crude virus diluted $1/10^4$ in baby mouse brain extract (BMBE) was 0.05 $\mu\text{g.}$ /ml. in each of three determinations. The titre was much lower (10 $\mu\text{g.}$ /ml.) in the presence of normal rabbit serum (Table 2).

Table 2. *Titre* of phospholipase A against MVE virus*

Medium and dilution	Crude virus ($\mu\text{g.}$ /ml.)	Purified virus ($\mu\text{g.}$ /ml.)
Undiluted	0.05	0.1–2.0
Undiluted + BMBE†	0.05	0.6
Undiluted + NRS‡	—	6.0
Diluted 10^{-4} in BMBE	0.05	0.05
Diluted 10^{-4} in NRS	10.0	> 30.0

* Concentration of phospholipase which destroyed 2 logs of infectivity in 30 min. at 37°.

† BMBE, an emulsion of normal baby mouse brain.

‡ NRS, normal rabbit serum.

The titre against undiluted purified virus varied between 2.0 and 0.1 $\mu\text{g.}$ /ml. (mean 0.9 ± 0.78 s.d.) over a series of 10 experiments. Purified virus is known to be relatively unstable in the absence of either serum or normal brain extract, and

although the controls in these ten experiments were satisfactory, the extremely wide variation in endpoints was ascribed to the instability of purified virus. As with crude virus, so here the enzymic action was inhibited by normal rabbit serum. The above results were obtained with boiled preparations of the enzyme. If instead of being boiled the purified phospholipase was used unheated, the titres against pure and crude virus were about tenfold higher.

A mixture of phospholipase (10 $\mu\text{g./ml.}$) and pancreatic ribonuclease (0.1 $\mu\text{g./ml.}$) was used to inactivate crude virus. The inactivation was slightly greater than with phospholipase alone (Table 3), but the product still yielded 'RNA' with a titre higher than the titre of treated virus from which it was derived. An incidental finding was that phospholipase A even in concentrations of 1 mg./ml. was harmless to the chick embryo when inoculated on to the chorioallantoic membrane. This enzyme is the active agent in certain snake venoms, and relatively small amounts were lethal when injected into chick embryo itself.

Action of phospholipase A on 'RNA'

Standard infective 'RNA' was treated with phospholipase A as described for virus. One batch of enzyme gave an end-point (destruction of 2 log of infectivity) of 'RNA' at 100 $\mu\text{g./ml.}$ and a second batch at 1 mg./ml. Each titration had a characteristically flat end-point, an indication that the destruction of 'RNA' may have continued after dilution of inoculum and after inoculation into eggs. Baby mouse brain extract (BMBE) had previously been found to be a diluent in which 'RNA' was relatively stable. However, in the presence of BMBE, 'RNA' was readily inactivated by phospholipase A, and the enzyme had an end-point at 0.05 $\mu\text{g./ml.}$

Table 3. *Infectivity remaining after treatment of crude MVE virus with enzymes*

Mixture incubated 37°, 30 min.	Titre of virus	Titre of 'RNA'*
Virus alone	7.0	4.6
Virus + phospholipase A (10 $\mu\text{g./ml.}$)	2.3	4.2
Virus + RNase (0.1 $\mu\text{g./ml.}$)	7.2	5.0
Virus + phospholipase (10 $\mu\text{g./ml.}$) + RNase (0.1 $\mu\text{g./ml.}$)	1.2	2.7

* 'RNA' made by the action of phenol on the corresponding mixture which had been incubated at 37° for 30 min.

Action of ether on virus and 'RNA'

At 4° ether decreased the infectivity of crude MVE virus by about 3 log in 5 min. (Table 4). No further infectivity was lost between 60 min. and 18 hr. Thus 0.1 % of the original crude virus was resistant to treatment for 18 hr. with ether at 4°. This resistant virus was studied. Ether resistance was phenotypic and not genotypic; this was shown by an experiment in which two serial limit-dilution passages of the treated virus were made in eggs and the resultant virus used to infect suckling mice. The progeny was as susceptible to ether as was the original stock virus.

Ether-resistant virus was not inactivated by ribonuclease, but some preparations were rather unstable at 37°. It was not clear whether this was intrinsic instability of ether-resistant virus, or due to the continued action of ether at 37°. Virus partly inactivated by ether was treated with phenol or deoxycholate to yield 'RNA'. The

titre of the yield was similar to that from the original stock virus (Table 4). Ether-resistant virus was completely inactivated by phospholipase A but the enzyme did not decrease the titre of potential infective 'RNA' (2.2 log compared to the control figure 2.5 log). Purified MVE virus was more completely inactivated by ether than was the crude virus, but again the reaction did not decrease the potential yield of 'RNA' (Table 4). Standard infective 'RNA' was normally freed from phenol by washing with ether. A sample of infective 'RNA' before and after such washing had titres 5.0 and 5.2 log, respectively, which showed that ether had not decreased the infectivity of 'RNA'.

Table 4. Action of diethyl ether on MVE virus at 4°

	Duration of treatment	Titre of virus	Titre of 'RNA'*
Crude virus	0 min	7.0	4.5
	5 min.	4.2	—
	60 min.	3.5†	4.3‡
	18 hr.	3.6	4.4
Purified virus	0 min.	10.4	—
	18 hr. alone (control)	8.3	4.0
	18 hr. with ether	1.2	3.9

* 'RNA' made by the action of phenol on the ether treated virus.

† Not destroyed by RNase (0.1 µg./ml. 37°; 30 min.).

‡ Destroyed by RNase (0.01 µg./ml. 37°; 30 min.).

Action of butanol, chloroform and lysolecithin on virus and 'RNA'

Butanol decreased the titre of crude virus from 7.6 log to zero in 15 min. at 4°. Some infective 'RNA' could still be recovered even after prolonged treatment of virus (Table 5). Butanol alone (60 min.) was without effect on the infectivity of undiluted 'RNA'; but a mixture of butanol and baby mouse brain extract destroyed 'RNA', decreasing the titre from 5.2 to 1.5 log in 60 min. at 4°, or from 5.2 log to zero in 30 min. at 20°. This finding suggested one possible explanation of the low yield of 'RNA' from butanol treated crude virus (Table 5).

Chloroform. When exposed to chloroform the titre of crude virus fell from 7.0 to 1.8 log after 2½ hr. 'RNA' was recovered from the inactivated virus, and had a titre of 4.4 log.

Table 5. Action of butanol on MVE virus (60 min., 4°)

Reaction mixture	Infective titre of reaction mixture	Titre of 'RNA'*
Crude virus alone	7.6	5.0
Crude virus + <i>n</i> -butanol	0.0	0.8
Pure virus alone	8.4	4.2
Pure virus + <i>n</i> -butanol	1.3	3.0

* 'RNA' made by the action of phenol on the final reaction mixture.

Lysolecithin. This agent was allowed to act at 37° for 30 min. The 2 log destruction end-point against MVE virus was at 500 µg. lysolecithin/ml. It was not clear, however, whether the lysolecithin itself was acting, or whether the activity was due

to contaminating phospholipase remaining from the process of manufacture of lysolecithin. Approximately 100 parts phospholipase/million could have produced this result.

DISCUSSION

The infectivity of both crude and purified MVE virus was decreased by purified phospholipase A and by diethyl ether, chloroform and *n*-butanol. The effective action was clearly on the virus preparation and not on the host chorioallantoic membrane, because dilution of treated virus did not restore infectivity. The interpretation of the destruction of virus by the phospholipase A preparation depends on an assessment of the purity of both the enzyme and the virus. Any contaminating enzyme in the phospholipase A must have survived boiling and few known enzymes are so heat stable. The most likely one would be an alkaline ribonuclease. As the virus particle is known to be stable to pancreatic alkaline ribonuclease, the virus inactivating principle in the preparation was most probably phospholipase A. The purity of the virus could not be estimated accurately, but the purified product seemed to be largely viral substance (Ada *et al.* 1961). Although crude MVE virus was grossly impure from a chemical point of view, the pattern of destruction of crude virus by phospholipase A was not obviously different from that of purified virus. These considerations suggested that the enzymic inactivation of virus was due to the cleavage by phospholipase A of fatty acid(s) from viral phospholipid(s). Lack of sufficient purified MVE virus has so far prevented search for split products.

The three solvents may have acted on the virus by attacking lipid components but there is as yet no direct evidence for this view. The rapid and complete inactivation of virus by *n*-butanol may be related to its reported efficiency in extracting phospholipids (Morton, 1950). The actions of diethyl ether and deoxycholate were studied separately by Andrewes & Horstmann (1949) and Burnet & Lush (1940), and the results were compared by Sunaga *et al.* (1960), who added several findings and concluded that the two agents were strikingly similar in their actions on viruses. Our work did, however, show one difference: while higher concentrations of deoxycholate liberated infective 'RNA' from virus (Anderson & Ada, 1959*b*), an aqueous solution of ether did not do so.

When we considered the significance of our results, the possibility that enzyme or solvent opened the virus particle and exposed the generic RNA to ambient ribonuclease was discarded, because higher titre 'RNA' could be recovered from the treated virus. The simplest interpretation and one most in line with current ideas, is that an intact phospholipid component on the virus particle is necessary during infection of the chorioallantoic membrane. An intact lipid envelope might enable the virus to penetrate the lipid layer of the surface of the host cell; or lipid components might be necessary for the integrity of specific receptor sites on the virus, which might function during entry into the host cell or during the early intracellular reactions of the virus. Another possibility cannot yet be eliminated. It is that lipid forms part of the mechanical structure of the virus particle although it does nothing specific in the process of infection of the host cell by virus. On this view enzymic or solvent alteration of viral lipid would distort viral architecture in such a way as to render the virus non-infective.

The small proportion of virus particles which resisted the action of ether at 4° was phenotypically but not genotypically distinct from the majority. This might suggest that the viral component attacked by ether was not directly genotypically determined, that it was not synthesized or incorporated under the immediate direction of the viral genome. It is tempting to believe that this ether-sensitive component is lipid, and indeed host lipid, and that it becomes wrapped around the virus particles just as the latter leave the host cell.

Because of the high concentration of phospholipase required to attack 'RNA', and also because of the differences of the titre between batches of phospholipase, it seemed possible that the action on 'RNA' was mediated not by the enzyme phospholipase A but by a contaminating heat stable ribonuclease. Previous titrations of pancreatic ribonuclease had shown a 2 log destructive end-point at 0.0007 µg. ribonuclease/ml. (Anderson & Ada, 1959*a*). Thus 7 parts of such ribonuclease/million in the first batch of phospholipase, or 0.7 parts/million in the second batch, would have been sufficient to account for this destruction of 'RNA' by the preparation of phospholipase A used. It is not suggested that the active agent in the preparation of phospholipase was precisely similar to pancreatic ribonuclease, but only that it was able to attack 'RNA' sufficiently to destroy infectivity. If this be accepted, it follows that the 'RNA' is insusceptible to phospholipase A, and presumably lacks those phospholipid components which have been postulated as necessary for infectivity of the intact virus. The resistance of 'RNA' to the three solvents also supports this conclusion. The destruction of 'RNA' by mixtures of normal baby mouse brain emulsion with either butanol or phospholipase suggests that the latter two reagents liberated, from BMBE, factors destructive for 'RNA'. It is pertinent to inquire whether these two factors may be identical, and whether they are ribonucleases.

In conclusion, we are inclined to adopt the second of the three alternatives discussed above, and to believe that MVE virus has one or more specific surface groups containing phospholipid, and that these play a vital role during infection of the host cell. Since 'RNA' is much less susceptible to phospholipase A and solvents than is intact virus, we must assume that 'RNA' neither possesses such groups nor needs them to effect entry into the susceptible cells of the chick embryo.

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Activation of one Tobacco Necrosis Virus by Another

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SUMMARY

Preparations of the Rothamsted culture of tobacco necrosis virus always gave two and usually three zones when centrifuged in sucrose density gradients. The top zone consisted of polyhedral particles with a sedimentation constant of 50S, the middle zone of larger polyhedral particles with 116S, and the bottom zone when present, consisted of the 50S particles aggregated in groups of 12. Neither the small particles (50S) nor their aggregates were infective when inoculated either to tobacco or French bean plants, but they became so when inoculated together with the large particles (116S). The small and large particles are serologically unrelated and seem to be different viruses, one of which depends on the other for some process that allows it to multiply to detectable amounts.

The Rothamsted culture produces local lesions of different sizes in French bean. Virus isolated from single large lesions and passed through a succession of single lesions gave, when bulked in tobacco, preparations containing up to 500 large to 1 small particle. Evidence is given which suggests that the few small particles were acquired as contaminants when the virus was bulked in tobacco, and it seems probable that small particles would not be produced in leaves infected only with the large particles. Virus obtained from small lesions when bulked gave preparations containing particles of both sizes, with ratios of large to small particles up to 1:10. Inocula of large particles produced only large lesions, whereas mixed inocula produced large and small lesions, in proportions which depended on the ratio of the two kinds of particles in the inoculum. Two different tobacco necrosis viruses activated the small particles, but tobacco mosaic and some other viruses did not.

Particles of the two activating viruses differed in size and in their stability when negatively stained in phosphotungstate. When fixed and negatively stained they appeared to be angular, but much less so than when shadowed. Small particles tended to pack in regular arrays, and one type of packing suggested that they have a fivefold axis of symmetry.

INTRODUCTION

The group of tobacco necrosis viruses (TNV) contains several serologically unrelated viruses (Bawden, 1941) which often occur in the roots of normal looking plants and are considered to be soil borne (Smith & Bald, 1935). The 'Rothamsted culture' of tobacco necrosis virus (RTNV) described by Bawden & Pirie (1945, 1950) differed from other members of the TNV group and from other 'spherical' plant viruses in several respects, but particularly in its behaviour when purified. Partially purified preparations were highly infective and serologically active and always

contained particles of at least two sizes, whereas preparations purified to the state of crystallizing consisted almost entirely of small particles, then thought to be about 17 m μ in diameter, and had relatively little infectivity, though they were still serologically active. Bawden & Pirie (1950) therefore suggested that only the large particles were infective and that the small ones, also nucleoprotein, might be derivatives from, or by-products of, the multiplication of the large particles.

Recently one of us (Kassanis, 1960) isolated from the roots of an apparently healthy tobacco plant growing in the greenhouse a TNV serologically related to RTNV but which, when examined in the electron microscope, seemed free from small particles. This suggested that the original culture of RTNV although derived from a single lesion might have been a mixture of two viruses. New attempts were therefore made to see whether the two kinds of particles could be separated by culturing from single lesions. A few of the cultures seemed to contain only large particles, but most contained both large and small particles, in different proportions in different cultures. Preparations of small particles only were obtained by centrifuging purified RTNV in sucrose density gradients. These were not infective, and leaves inoculated with them produced no lesions and yielded no small particles. Although unable to multiply by themselves, the small particles are produced when inoculated to plants also infected with the large particles.

METHODS

Single-lesion isolates were obtained from an inoculum of RTNV supplied by Mr F. C. Bawden, which was diluted so that it gave only a few lesions on tobacco leaves (*Nicotiana tabacum* L., White Burley var. Judy's Pride); about 1 week after inoculation selected lesions were cut from the leaves, macerated and inoculated separately to healthy tobacco plants. This process was repeated until each isolate had passed successively through six single lesions. Tobacco necrosis viruses do not become systemic, but give only necrotic lesions on the inoculated leaves of susceptible plants. The single lesion cultures had therefore to be bulked to provide enough inoculum to inoculate the large number of leaves needed to prepare virus in quantity. This was done in two stages. In the first, the extract from each single lesion was diluted in water and inoculated to as many tobacco leaves as possible. Sap from these leaves was stored in small equal samples at -20°C , and used to inoculate further tobacco leaves in the second stage. The sap from these leaves, again stored in equal samples at -20°C , was inoculated to the batches of plants used to prepare virus in quantity. In the summer, plants were covered with a black cloth for a few days before inoculation to increase their susceptibility. 'Celite' was added to all the inocula to increase the number of lesions.

The virus was purified by the methods described by Bawden & Pirie (1957). Inoculated leaves, harvested about 1 week after inoculation, were passed through a domestic mincer and the pulp squeezed through muslin. The pulp was then extracted in a blender with a volume of water equal to the expressed sap, and squeezed again. (The pulp contained more than half as much virus as the sap.) The combined sap and extract was stored frozen at -20° . When required, it was thawed and allowed to stand for 24 hr. at 20° to denature normal proteins and to destroy the virus-inactivating system it contains (Bawden & Pirie, 1957), after

which it was centrifuged at 8000 *g* for 5 min. The resulting brown supernatant fluid was then centrifuged at 75,000 *g* for 3 hr. The pellet was suspended in a small volume of water and left overnight at 5° before centrifugation at 8000 *g* to remove insoluble material. A further cycle of high- and low-speed centrifugation gave preparations which were colourless, strongly light scattering and highly infective.

Infectivity was assayed on the primary leaves of French bean (*Phaseolus vulgaris* L., var. Prince); 8 half leaves were rubbed with each inoculum.

Antisera were prepared in rabbits, by two intravenous injections of purified virus, separated by an interval of about one month. Serological tests were made either by precipitation tests in narrow tubes or by double diffusion precipitation in agar/water or agar/phosphate gels at pH 8. The antiserum to RTNV was that used by Bawden & Pirie (1945, 1950).

Rate zonal centrifugation in sucrose density gradients (Brakke, 1953) was used to separate the particles of different sizes using the techniques described by Harrison & Nixon (1959). After centrifugation, the zones were removed with a micropipette which could be lowered mechanically into the gradient columns while they were being observed under vertical illumination against a black background. The samples were then dialysed against distilled water before counting the particles, or assaying infectivity or serological activity.

Particles were counted by a modification of the technique described by Nixon & Fisher (1958). Virus samples were mixed with serum albumin, polystyrene latex suspension, and neutral sodium or potassium phosphotungstate before they were sprayed on the collodion-covered electron-microscope grids. In this way normal plant protein and microsomes could be distinguished from the small particles; also, the mounts could be examined and photographed in the electron microscope immediately, without the delay associated with the more usual shadowcasting. The actual counting was done on prints at about $\times 75,000$ to facilitate discrimination between large and small particles (Pl. 2, fig. 8). The electron microscope used was a Siemens Elmiskop I, and magnification calibrations were derived from a 70μ hole in a platinum aperture, measured with a light microscope. Duplicate calibration measurements were usually within 1 %, and we think it unlikely that our magnifications in routine work were in error by more than 3 %.

Particle diameters were measured with a calibrated light microscope from the original photographic plates. Usually 30 particles were measured from each plate, and images of shadowed particles were always measured at right angles to the shadowing direction, regardless of the orientation of any angular outline that was visible. All the large particles, and especially those from TNVb (see later) preparations, were heavily flattened when air dried on collodion films and then shadowed in the usual way; TNVb particles also tended to break up when mixed with neutral phosphotungstate and sprayed on the carbon-coated mounts. Both these undesirable effects could be greatly decreased by treating the virus preparations with 1–2 % formaldehyde for a few hours, and this was done when electron micrographs suitable for measurements were needed.

RESULTS

Separation of large and small virus particles by single-lesion isolation

Out of a total of ten single-lesion cultures prepared from the original inoculum of RTNV, four gave virus preparations which were thought at the time to contain only the large particles and six gave mixtures of large and small particles in different ratios. From these ten isolates three were selected that differed from each other in the type of lesion produced, although all reacted with antiserum to the original RTNV. The three isolates were: TNVs, containing one large to about ten small particles; TNVa and TNVb, both of which apparently contained only large particles when newly made preparations were examined with the electron microscope. However, after preparations of the last two isolates had been stored for some time, small particles were detected in them. Thus, a sample of TNVb, which initially contained fewer than one small to 500 large particles, had 3 months later about one small to 50 large particles. We think that this reflects the different stabilities of the large and small particles; when stored the preparations became enriched with the more stable small particles simply because some of the less stable large ones denatured. Small particles could also be detected in preparations derived from TNVa and TNVb by reculturing these isolates two or three times in tobacco leaves. As we shall show, this contamination might easily have come from the roots of the apparently healthy tobacco plants used for propagating and reculturing the viruses. The isolate TNVa was similar to the one found by Kassanis (1960) and referred to in the Introduction. The large particles in it were more stable than those in TNVb, although in addition to these stable large particles, preparations of TNVa usually also contained some TNVb particles, and some small particles in about the same ratio as found in isolate TNVb. The lesions produced on beans by TNVa appeared a day earlier, were darker in colour and spread more slowly, than those produced by TNVb. The lesions produced by TNVa and TNVb were uniform in size, in sharp contrast to the different sizes usual on leaves inoculated with RTNV or TNVs.

Careful examination of many preparations in the electron microscope has convinced us that small particles never appeared as a result of breakdown of large ones, for images which might represent an incomplete breakdown of this sort have never been seen, although partly broken large particles were very common, particularly in unfixed negatively stained preparations of TNVb. Such breakdown seemed to be associated with the phosphotungstate, for when the particles were treated with formalin for a few hours before adding the phosphotungstate, only a few partly broken ones were seen, and the pictures resembled those obtained with TNVa (Pl. I, figs. 3, 5).

Some experiments were made to try and explain the reasons for our failure to produce pure cultures of the large particles. In one of these experiments, extracts were made from the roots of apparently healthy tobacco and of other species of plants commonly grown in the Rothamsted glasshouses. These extracts were all highly infective when inoculated to bean or tobacco leaves, and produced typical tobacco necrosis lesions. Electron microscopy showed that the extracts contained up to 1×10^{11} small and 0.3×10^{11} large particles/ml., or between $\frac{1}{10}$ th and $\frac{1}{100}$ th of the amount to be expected in inoculated leaves used for making virus preparations.

Tobacco necrosis viruses have often been detected at Rothamsted in leaves of plants systematically infected with other viruses. This suggested that a further test might be made to see whether any of the relatively large amounts of TNV commonly found in the roots of apparently healthy plants could escape into the leaves. When sap from symptomless and apparently healthy tobacco leaves was treated as if it had contained TNV, on two out of three occasions the preparations gave an average of 2 lesions/leaf when inoculated on to bean leaves. No particles were found in these extracts with the electron microscope, but detection by electron microscopy is very much less sensitive than infectivity tests. The lower limit for detection is in the region of 10^9 particles/ml., a concentration which gives many lesions/leaf when inoculated to beans under our conditions. TNV in small amounts has also been found in the leaves and flowers of naturally infected *Primula obconica* (Bawden & Kassanis, 1947) and in the leaves and runners of strawberry plants grown under glass (Fulton, 1952). There is thus good evidence that the roots of many apparently healthy plants contain quite high concentrations of TNV, and that significant quantities of this virus can appear in the leaves, even though these remain symptomless. Our failure to obtain and retain pure cultures of large particles is hardly surprising in these circumstances.

Separation by centrifugation

After we had failed to obtain single-lesion cultures containing only small particles, attempts were made to separate the small particles from the large by centrifuging preparations of TNVs in sucrose density gradients. After centrifuging such preparations for 2 hr. at 25,000 g, two and sometimes three, light-scattering zones were formed. When dialysed and examined in the electron microscope, the top zone, as expected, consisted almost wholly of small particles, the middle zone of large and small particles, and the bottom zone of aggregates of small particles (to be discussed later). Separation was never quite complete. Typical ratios of small to large particles in the three zones were 100:1, 0.5:1 and 250:1 when counted in negatively stained droplet traces with the electron microscope. Because of the rapid increase of light-scattering power with increasing particle size, the bottom zone, when present, was often the most easily seen, although it contained the fewest particles. The material from the top zone was usually non-infective, so that the few large particles present in it were probably damaged and were perhaps without their nucleic acid. Damage of this kind would account for their presence in the zone of small particles each of which was only one-half to one-third of the weight of a single complete large particle, assuming the densities of the two kinds to be equal. The middle zone from the gradients was too heavily contaminated with small particles to form a useful source of the large ones, which could be obtained cleaner from preparations of TNVa and TNVb. Although it is difficult to sample one zone through another without contaminating the particles from the lower zone with some from the upper one, much better separations have been obtained in our laboratory with other viruses by using the same techniques. We think the middle zone may be contaminated by partly broken aggregates, not heavy enough to sediment with the bottom zone of complete aggregates but too heavy to remain the top zone of single small particles. Such partly broken aggregates would presumably be unstable and might break down to single particles during the subsequent dialysis.

By measuring the depths to which the zones sedimented in the sucrose gradients and comparing these measurements with the depth to which tobacco mosaic virus sedimented in another gradient centrifuged at the same time, the sedimentation constants could be estimated. Assuming the value for tobacco mosaic virus to be 187S (Lauffer, 1944), our values for the three TNV zones were 45S, 116S and 216S (Kassanis & Nixon, 1960). These figures agree well with values of 50S and 116S kindly obtained for us by Dr R. Markham in the analytical centrifuge with a 0.3% virus suspension. When aggregates previously separated in a sucrose gradient were run in the analytical centrifuge, values of 49S and 222S were obtained on a 0.24% suspension, suggesting that some aggregates must have broken into single particles during dialysis and storage before analytical ultracentrifugation. When sap from tobacco leaves (macerated with some KCN) infected with TNVs was run in the analytical ultracentrifuge, a total of six sedimenting boundaries was identified. Plate 3, fig. 14, is a typical picture; the successive peaks are, from right to left; (a) 2 peaks of normal plant protein; (b) of small particles; (c) 2 peaks of microsomes; (d) large particles. No boundary for the aggregated small particles with a sedimentation constant of 222S could be found in infective sap. This makes it appear likely that the aggregates formed during purification. Our values for the sedimentation constants also agree well with those of Ogston (1942) who used preparations of RTNV made by Bawden & Pirie (1942). Only one of the preparations examined by Ogston showed boundaries for both 116S and 240S components, although the preparations of TNV (apparently a mixture of RTNV with another virus) made by Pirie, Smith, Spooner & McClement (1938) contained all three, the 116S component in the crystalline fraction and the two others in the amorphous fraction. A possible explanation of Ogston's results is that all but one of the preparations he examined contained as their large component a high proportion of TNVb particles, which are less stable than TNVa and would therefore tend to be eliminated during the preparation of highly purified samples.

The size and shape of the particles

Negatively stained small particles looked much smaller than shadowed ones. In this respect the small particles resemble three soil-borne viruses with polyhedral particles (Harrison & Nixon, 1960). The difference was presumably caused by the stain penetrating the outer parts of the particles, and perhaps also by slight residual flattening and consequent overestimation of the diameter of the formalin-fixed shadowed particles, which were air dried without the support of a surrounding film of phosphotungstate. By contrast, both the isolates of large particles proved to be remarkably uniform in diameter when examined in negatively stained and in shadowcast mounts (Table 1). Shadowed large TNV particles often seemed angular, and when this happened the outlines were usually hexagonal (Pl. 1, figs. 2, 4). Some particles had pointed and some flat-topped shadows, but neither the angular outlines nor the shadow shapes appear consistently enough to permit any useful deductions to be made about the exact shape of the large TNV particles, except the very general one that they are almost certainly polyhedral. This impression is supported by the somewhat angular appearance of the large particles in negatively stained preparations (Pl. 1, figs. 1 and 3). Small particles also looked angular when fixed with formalin before shadowing, and tended to pack into regular arrays in

negatively stained mounts (Pl. 2, fig. 7). Much of the apparent angularity in negatively stained preparations is an illusion caused by regular packing.

By studying the various ways in which the small particles packed some deductions can be made about the symmetry of each individual particle. We are indebted to Mr G. Brown of the Pedology Department at Rothamsted for the interesting suggestion that the appearance of an angle of 72° ($360^\circ/5$) between the lines of particles in one type of packing (Pl. 2, fig. 7) indicates that each of the small particles may have a fivefold axis of symmetry. The presence of fivefold axes of symmetry is one of the features of the (532) symmetry that has recently been demonstrated by X-ray diffraction and electron microscopy in some other virus particles. This evidence for fivefold symmetry in the small TNV particle suggests that it may possibly have similar symmetry.

Table 1. *The size of tobacco necrosis virus particles (TNV) estimated from electron micrographs made by two different techniques*

Material	Diameter (m μ)
TNVa	
Stained	29.88 \pm 0.60
Shadowed	29.69 \pm 0.36
TNVb	
Stained	25.05 \pm 0.44
Shadowed	26.83 \pm 0.41
Small particle	
Stained	14.28 \pm 0.19
Shadowed	21.21 \pm 0.60

The aggregates which formed the bottom zone after centrifugation in sucrose were visible only in negatively stained mounts, and even in these they sometimes collapsed into groups of small particles (Pl. 2, fig. 8). Aggregates occurred in three orientations, and the images can all be accounted for by assuming that each aggregate consisted of twelve small particles, arranged in two rings of five placed back to back, with an additional particle placed at the centre of each ring (Pl. 1, fig. 6). The most common number of particles found in the groups thought to represent collapsed aggregates was twelve, which agrees well with the conclusion about the structure of the aggregates reached from micrographs of the aggregates themselves. Small particles taken from broken aggregates were indistinguishable in size and appearance from those in preparations of unaggregated small particles taken from the top zone after centrifugation in sucrose density gradients. Neither aggregates nor groups thought to represent collapsed aggregates were found in shadowed mounts, even when aggregates were common in negatively stained mounts made from the same virus preparations. Presumably the aggregates were destroyed by drying them without a supporting film of phosphotungstate and the resulting small particles were scattered over the mount in the remaining liquid film, so that no discrete groups could be recognized. It is interesting to note that the aggregate structure also has the (532) symmetry already discussed.

Infectivity tests

When the zones in the density gradient tubes were clearly separated after centrifugation and the sampling carefully done, only material from the middle zone was infective and produced lesions when inoculated to bean or tobacco leaves. Leaves inoculated with preparations of small particles from the top zone not only failed to produce lesions, but no virus-like particles could be detected, either by serology or electron microscopy, in clarified sap from the leaves or after the sap had been subjected to the procedures used to purify and concentrate the virus. In contrast, extracts from leaves inoculated with a mixture of small particles from the top zone of a sucrose density gradient and large particles from TNVa or TNVb preparations, contained small and large particles in roughly equal numbers (Table 2). Similarly, aggregates from the bottom zone did nothing alone, but multiplied when inoculated together with TNVa or TNVb. Old preparations of TNVa, substantially free from the less stable TNVb particles, were as efficient as fresh preparations in aiding the multiplication of small particles. Both the large particles therefore

Table 2. *The ratio of large to small particles in virus preparations purified from tobacco plants inoculated with large particles of tobacco necrosis (TNV) alone or in mixtures with the small particles*

Virus inoculum*	Ratio:large/small				
	Expt....1	2	3	4	5
TNVb (large)	—	—	20	83	—
TNVa (large)	12.5	20.6	—	—	13
Large† + small	1.3	0.9	2.2	0.2	0.7
Small	No virus detected.				

* The leaves were inoculated with the aid of 'Celite'; the concentrations of inoculum in mg./l. for the large and small particles were, respectively: Exp. 3, 4 and 100; Exp. 4, 40 and 40; Exp. 5, 40 and 100.

† The large particles used in Exps. 1, 2 and 5 were TNVa and in Exps. 3 and 4 TNVb.

activated the small ones. The ratio of the two kinds of particle in the inoculum was not the only factor that determined the ratio in the product, for when young and old tobacco leaves were inoculated with the same mixed inoculum and harvested separately, the ratio of small to large particles was 50:1 in the virus preparation made from young leaves and 16:1 in a similar preparation made from old leaves, suggesting that young leaves were relatively more susceptible than old leaves to the small particle. From these results we conclude that, whereas the large particles can infect and multiply unaided, the small ones cannot, and multiply only when they are in leaves together with the large. If this be so, then it should also be possible to obtain pure isolates of the large particles which can be cultured repeatedly without becoming contaminated with small ones. This we were unable to do, for, as already described, many healthy looking tobacco plants contain TNV.

The results in Table 2 are from experiments in which the large and small particles were inoculated to tobacco plants simultaneously. The small particles were also activated when leaves were inoculated with the two kinds of particles at different times. For example the ratios of large to small virus particles in virus preparations

obtained from tobacco leaves inoculated with the same preparation of the two particles but at different times were: 0.2 when the leaves were inoculated with both particles simultaneously; 1.2 when the small particles were inoculated 2 days before the large (TNVb); 5.9 when the large particles were inoculated 2 days before the small; and 23 when inoculated with the large particles only. Most small particles were produced when a mixed inoculum was used, and more when the small particles were inoculated before the large than afterwards. No other tobacco necrosis viruses have been tested, but tobacco mosaic, lucerne mosaic, carnation ringspot, and tomato bushy stunt viruses all failed to make the small particles multiply.

Preparations of TNVa disrupted by phenol are highly infective (Kassanis, 1960). Phenol-disrupted preparations of the small particles, like the whole small particles, did not multiply in bean or tobacco plants. When, however, such preparations were mixed with TNVa or TNVb, either intact or phenol-disrupted, the small particles multiplied. In one such experiment the ratio of large to small particles was 2.4 in virus preparations made from tobacco plants inoculated with a mixture of phenol-disrupted small particles and TNVa, and 40 in preparations from plants inoculated with disrupted TNVa alone.

Inhibition of lesion formation

Tobacco and bean leaves inoculated with TNVs produced a mixture of small and large lesions, whereas TNVa, TNVb gave only large-size lesions (Pl. 3, figs. 11, 12). When inocula of TNVa or TNVb were mixed with different concentrations of small particles, the number of small lesions increased with increasing number of small particles in the inoculum (Table 3). In the experiment in Table 3 the total number of lesions remained about the same with all concentrations of small particles, but when the concentration of small particles in the inoculum increased much above 5 mg./l. the total number of lesions decreased (Kassanis & Nixon, 1960).

Table 3. *The infectivity of tobacco necrosis virus (TNVb) in the presence of different amounts of small particles*

Concentration of small particles (mg./l.)	TNVb = 0.8 mg./l.	
	Number of lesions on 8 half leaves of French beans	
	Large lesions	Small lesions
5	20	274
1.25	44	199
0.31	157	95
0.08	151	33
0	217	0

Freshly prepared TNVa or TNVb produced only large lesions, and behaved as if they contained only large particles, presumably because the fresh preparations had to be diluted considerably to give countable lesions, and this dilution lowered the concentration of small particles in the inoculum to a concentration below that needed to infect. Stored preparations, however, had to be diluted less to give the same number of lesions, and these gave some small lesions.

Serological tests

From plants inoculated with small particles only, nothing could be isolated that precipitated specifically with antiserum to TNVs (mixed particles), whereas virus prepared from plants inoculated with the small and large particles gave two lines in gel diffusion tests, showing that the two particles were serologically unrelated. Plate 2, figs. 9 and 10, show the results of two such tests. In both tests the three upper cups contained, from left to right, virus prepared from tobacco leaves inoculated with (a) small particles alone; (b) large particles alone; (c) the mixture. For Pl. 2, fig. 9, antiserum to TNVs was placed in the centre cup and, for Pl. 2, fig. 10, the antiserum was to large particles only. Fig. 10 shows only one precipitation line, and fig. 9 shows two lines near the top right cup which contains the virus produced from mixed inoculum. Similar results were obtained with TNVa and TNVb.

Table 4. *Dilution end points of antisera tested against homologous and heterologous viruses*

Antiserum	Antigen (at 0.04 g./l.)		
	Small particles (Top zone)	TNVa	TNVb
	End-point dilution		
TNVa	0 (1/5)*	1/160	—
TNVb	0 (1/5)	—	1/80
TNVs (mixture)	1/160	1/80	1/80
TNVs (mixture) absorbed with TNVa	1/160	0 (1/20)	—
TNVs (mixture) absorbed with TNVb	1/160	—	0 (1/20)

* The figures in parentheses show the highest concentration at which the antisera were tested.

The absence of any detectable serological relationship between the large and small particles was also demonstrated in the three lower cups in Pl. 2, fig. 9, which contained mixtures of large and small particles in different ratios, for the two precipitation lines cross. In Pl. 2, fig. 10, the same set of mixtures gave only one line when tested against an antiserum to large particles only. Precipitation tests in tubes also failed to show any common antigens between the small particles and either TNVa or TNVb (Table 4). Antiserum to either of the two large particles did not precipitate at dilutions greater than 1/5 with the small particles obtained from the top zone of sucrose density gradient columns, but each precipitated against preparations of the corresponding homologous particle. Also, the antiserum to TNVs (mixed particles), when absorbed with either of the two large particles TNVa or TNVb, still precipitated against preparations of small particles to the same dilution as it did when unabsorbed. So far we have not established a serological relationship between TNVa and TNVb.

DISCUSSION

The small particles evidently multiplied detectably only in the presence of the large particles; but our results do not show with the same degree of certainty that the large particles could multiply without producing any small ones. A reasonable explanation for the preparations of TNVa and TNVb containing a few small particles is that they became contaminated when propagated in tobacco plants, for we have shown how easily leaves can become infected from the heavily infected roots. However, there are other possibilities. First, although some single lesion isolates consist only of large particles, small particles might appear as mutants, for the possibility that the two kinds of particles are remotely related cannot be excluded. The fact that they are of different sizes does not in itself eliminate the possibility of a distant relationship between them, for such relationships have recently been shown between a number of rod-shaped viruses with different normal lengths (Bercks, 1960; Wetter, Quantz & Brandes, 1959). Alternatively, the association between large and small particles may be so close that it is not broken by the methods we have used. On this view repeated single-lesion isolation might merely alter the ratio in favour of the large particles, and the ratio would then gradually return to its previous value when the culture was propagated without continuous selection. This second possibility casts some doubt upon the ability of the large particles to function without the small; but until the contrary is proved we think it reasonable to assume that the large particles neither need the small ones nor directly produce them.

None of the properties of the small particles so far investigated suggests that they should not be called virus particles, for in some circumstances they infect and multiply. Like the large particles, they consist of nucleoprotein and, in common with other tobacco necrosis viruses, they contain from 1.7 to 2.0 % phosphorus (Bawden & Pirie, 1942). The difference in size means that each small particle has only about one-third to one-half of the volume of a large one and, assuming that the densities of the two kinds to be the same, each small particle will contain a correspondingly small amount of nucleic acid. The particles are smaller than other known spherical viruses and their inability to multiply on their own may reflect an inadequacy of nucleic acid and the need to 'borrow' some missing part from the large particles.

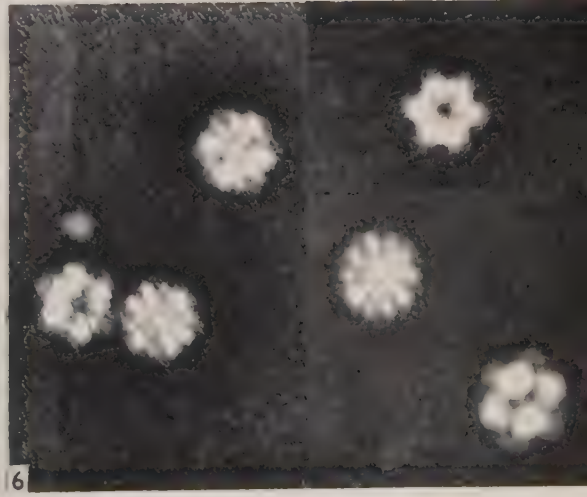
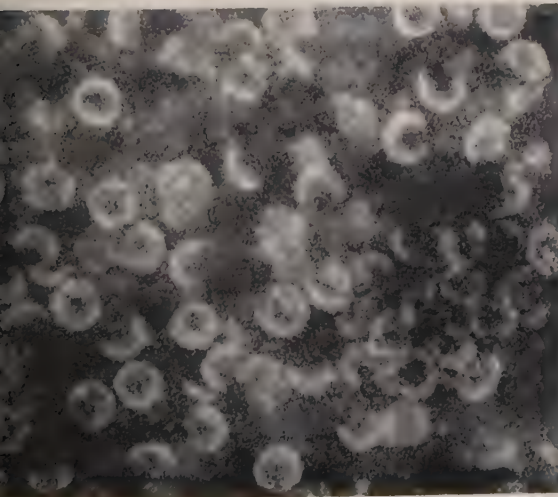
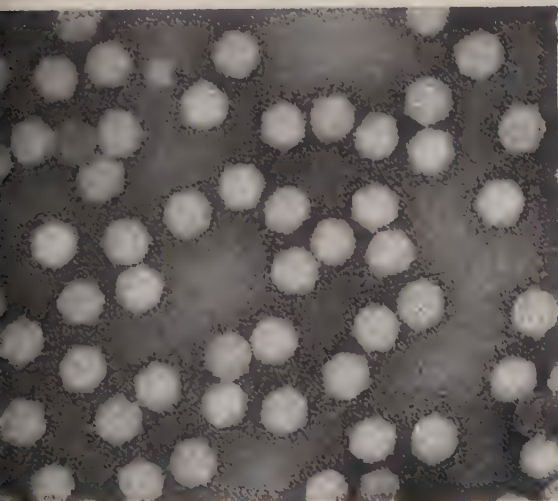
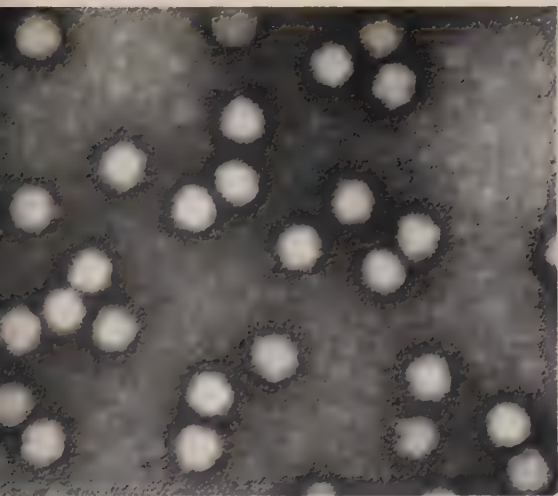
Finally the possibility must be considered that the small particles do multiply, but without causing any symptoms and without reaching concentrations high enough to be detected even by infectivity tests. In the presence of the large particles, the concentration of the small ones might rise to the concentration found in normal mixed cultures. There are other examples of one virus increasing the concentration of another (Rochow & Ross, 1955; Kassanis, 1961), but with these the increase is small compared with the amount reached by the one virus inoculated alone. If the small particles do multiply on their own, perhaps in the cells they enter at the time of inoculation, they do not reach even 1/1000th the concentration they reach in mixed cultures with the large particles, for if they did they would have been detected with the electron microscope. Movement from cell to cell, however, might be the ability conferred on the small particles by the large; but until there is evidence of multiplication in the initially infected cells, there is no good reason for assuming this, rather than any other, to be the critical stage aided by the large particles.

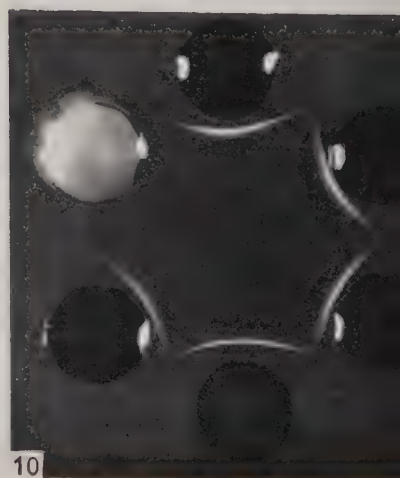
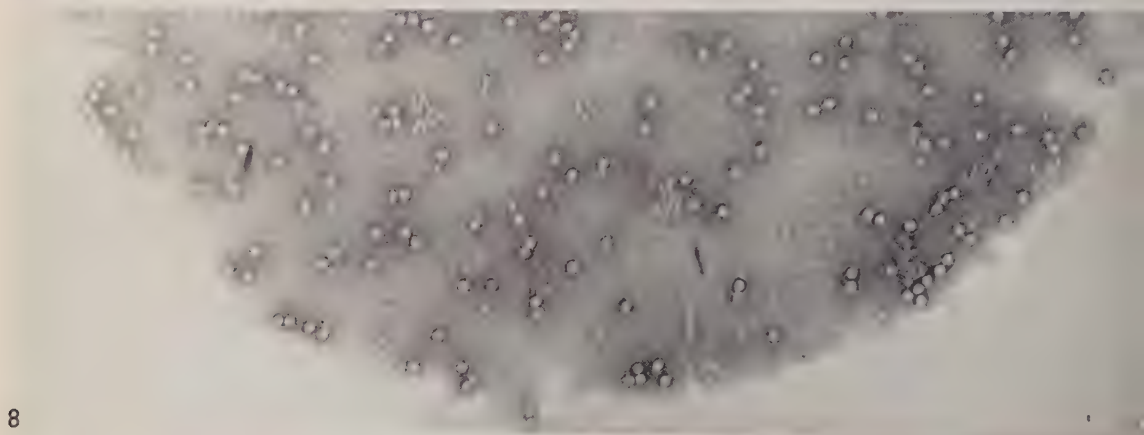
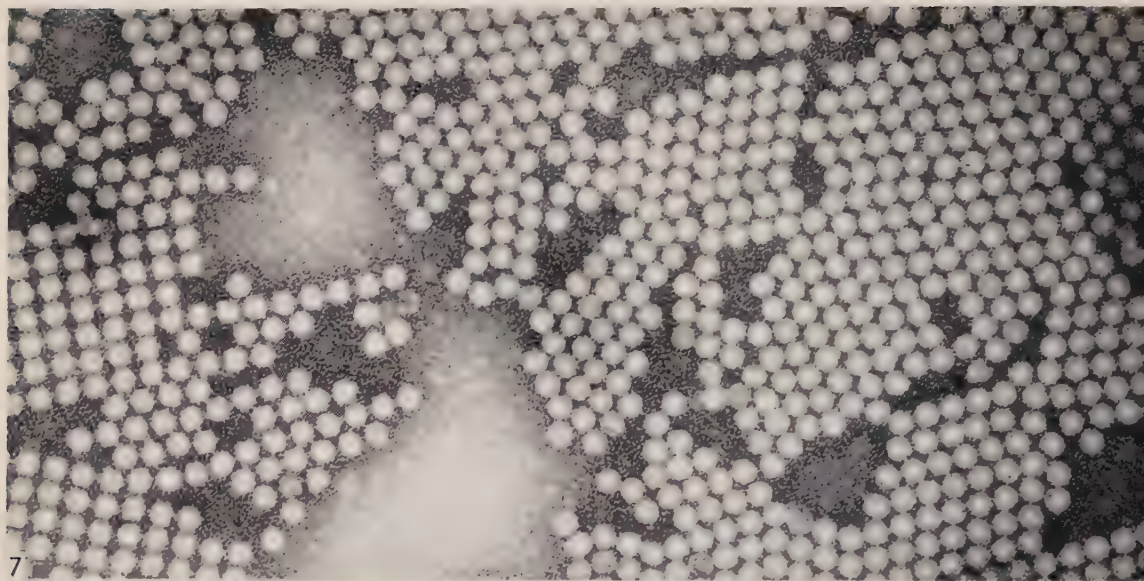
Further evidence, about possible relations between large and small particles and about the continued ability of the large virus to multiply without producing any small particles, is unlikely to be obtained until the viruses can be propagated in conditions free from the risk of contamination by tobacco necrosis viruses present in the roots and leaves of apparently healthy tobacco plants.

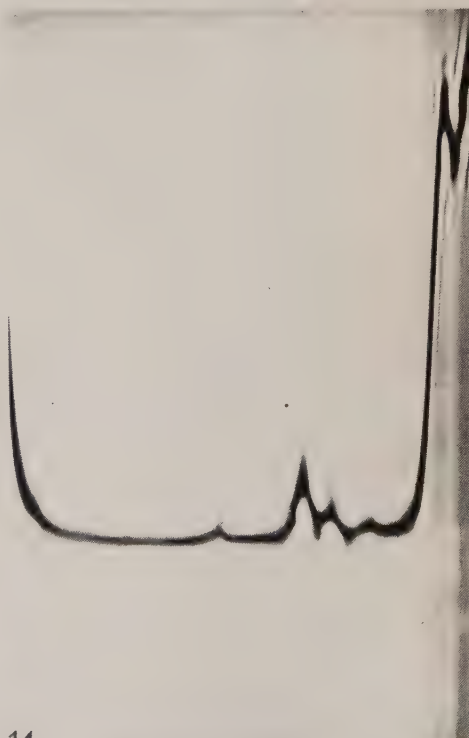
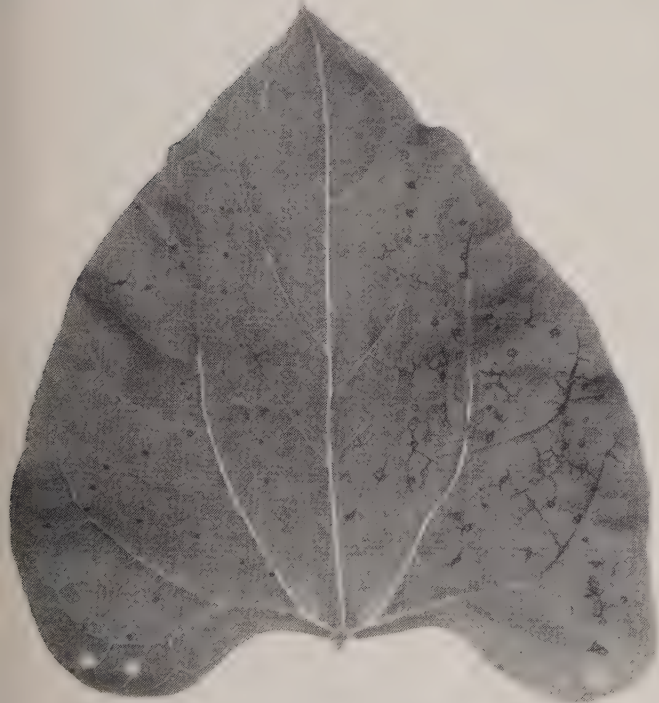
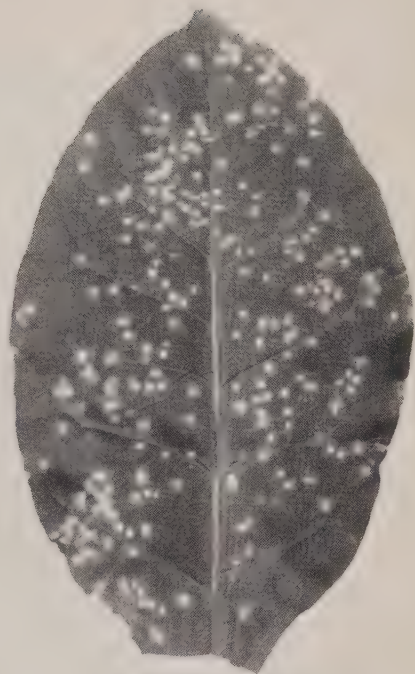
We are indebted to Mr R. D. Woods for his able assistance with the electron microscopy.

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EXPLANATION OF PLATES

PLATE 1

- Fig. 1. TNVa particles fixed with formalin and mounted in phosphotungstate. $\times 240,000$ approx.
Fig. 2. TNVa particles fixed with formalin and shadowed with platinum-iridium alloy. $\times 108,000$.
Fig. 3. TNVb particles fixed with formalin and mounted in phosphotungstate. $\times 240,000$ approx.
Fig. 4. TNVb particles fixed with formalin and shadowed with platinum-iridium alloy. $\times 108,000$.
Fig. 5. TNVb particles mounted in phosphotungstate without fixation. $\times 240,000$ approx.
Fig. 6. Aggregates, each consisting of 12 small particles, from the bottom zone of a preparation of TNVs after centrifuging in a sucrose density gradient. Mounted in phosphotungstate. $\times 240,000$ approx.

PLATE 2

- Fig. 7. Small particles from the top zone of a preparation of TNVs after centrifuging in a sucrose density gradient, mounted in phosphotungstate. Three different types of packing can be seen. Many particles on the right of the picture are packed in rows inclined at 72° , suggesting that they are oriented with a fivefold axis emerging at the centre of each particle. $\times 240,000$ approx.
Fig. 8. Part of a droplet trace made by a virus preparation (TNVa) from plants inoculated with large and small particles, mixed with phosphotungstate, showing large and small particles. Some of the large particles have been penetrated by the phosphotungstate and resemble those shown in Fig. 5. $\times 74,000$.
Fig. 9. Gel diffusion test in which the 3 upper cups contained from left to right: (a) virus preparation from plants inoculated with small particles; (b) large; and (c) large and small particles. The three lower cups contained virus preparations with different ratios of large and small particles, the central cup having more small particles than large and those on each side of it more large than small. The cup in the centre contained antiserum to both large and small particles.
Fig. 10. Gel diffusion test as in Fig. 3 but with antiserum to large particles in the centre.

PLATE 3

- Figs. 11, 12. Tobacco leaves inoculated with two kinds of particles; in fig. 11 the inoculum was a mixture of large and small particles, in fig. 12 the inoculum was large particles only.
Fig. 13. Bean leaf inoculated with two kinds of particles; the left half with large and small particles, the right with large particles only.
Fig. 14. Sedimentation pattern of sap from leaves infected with TNVs. The boundaries are, from right to left: two large peaks of normal plant protein, a very small peak with $S = 43$ (small virus particles), two larger peaks with $S = 58$ and 69 (microsomes), and a very small peak with $S = 108$ (large virus particles).

Correlation of the Vitamin Requirements with Cultural and Biochemical Characters of *Lactobacillus* spp.

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SUMMARY

Improved media are described for the study of the vitamin requirements of a wide range of *Lactobacillus* spp., some of which had not previously been grown consistently well. In general, the nutritional data are consistent within species and correlate well with the other differentiating characteristics of species. Also, the nutritional requirements were in agreement with results obtained with several species some years ago. Improved or abundant growth, not achieved in previous studies with several other species, resulted from the use of the more nearly optimal media developed in the present study. The growth of *L. bulgaricus* was prevented or inhibited by folic acid at 10 $\mu\text{g./l.}$

INTRODUCTION

Since the pioneer work of Snell & Strong (1939) on the assay of riboflavin with *Lactobacillus casei* var. *rhannosus* (American Type Culture Collection, ATCC7469) microbiological methods have come into general use for the determination of several other essential nutrients. Indeed, in some instances, as in the measurement of biotin, folic acid and vitamin B₁₂ in low-potency foodstuffs, no convenient alternative method is yet available. The accumulation of a large literature on the nutrition of the lactic acid bacteria has quickened an awareness of the complexity of nutritional interrelationships essential for enzymic activity, growth and its inhibition. An excellent, brief but comprehensive, survey of this subject was made by Snell (1952).

It has become increasingly apparent that requirements for essential metabolites, under carefully standardized optimal conditions, generally are characteristic for the species of a genus, even though sometimes an individual strain may behave exceptionally. For instance, Fildes (1923, 1924) differentiated members of the genus *Haemophilus* on the basis of their requirements for the 'X' and 'V' factors. More recently, in an extensive study of the genus *Bacillus*, Knight & Proom (1950) and Proom & Knight (1955) showed that the requirements for vitamins and amino acids

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were closely correlated with other species characteristics. Rogosa, Tittsler & Geib (1947) found a very high correlation of vitamin requirements with cultural and biochemical characteristics within the genus *Lactobacillus*. With 500 oral strains Rogosa *et al.* (1953) confirmed the results of a previous study (Rogosa *et al.* 1947) by again finding a high degree of correlation between nutritional requirements and the cultural and biochemical characteristics of species. However, certain species could not be tested for nutritional requirements because they were not cultivatable in any of the media then used; some of these organisms even may grow poorly and erratically in present-day widely used media. Among these species were *L. bulgaricus*, *L. lactis*, *L. helveticus*, *L. jugurti*, *L. acidophilus*, *L. delbrueckii* and *L. leichmanii* (Tittsler, Rogosa & Whittier, 1942; Rogosa *et al.* 1947). Except for *L. acidophilus*, these have not been regularly isolated from human or animal oral samples.

As a consequence of expanding knowledge, media for assay purposes have become plentiful, but media developed for specific assays with specific organisms may not be generally applicable for a wide range of species. The purpose of the present work, therefore, was to devise a medium suitable for the optimal growth of a number of species which grew poorly or not at all in any media used in earlier nutritional tests. Furthermore, since some species have already been systematically studied, an attempt has been made to determine the stability of their nutritional requirements and then the reliability of this character as a useful adjunct in the correlation of characteristics required for the adequate and realistic differentiation of species.

METHODS

Media. The composition of the basal medium used is shown in Table 1.

The solution of vitamin-free acid-hydrolysed casein (Table 1) was prepared from the product of Allen and Hanbury's Ltd., or from the 'vitamin-free, salt-free' casein hydrolysate (acid) of the Nutritional Biochemicals Corporation, by the method of Ford, Perry & Briggs (1958). Various charcoals (e.g. Norit A, Darco G60, the Grade 5 activated charcoal of Sutcliffe and Speakman Ltd.) were satisfactory. Additions of the cyclic amino acids, DL-tryptophan, L-tyrosine, DL- β -phenylalanine, L-proline and L-histidine were made exactly as indicated by Ford *et al.* (1958).

Table 1. *Composition of the 'complete' basal medium*

Quantities per litre final medium.			
Solution of vitamin-free acid-hydrolysed casein (see text)	100 ml. ~ 5 g.	Solution of mineral salts (see text)	10 ml.
Solution of vitamin-free pancreatic casein digest (see text)	100 ml. ~ 4 g.	Adenine; guanine; xanthine; uracil	each 5 mg.
Glucose	10 g.	Pyridoxal ethyl acetal hydrochloride	2 mg.
K ₂ HPO ₄ ; KH ₂ PO ₄	each 3 g.	Niacin; thiamine HCl; Ca pantothenate; riboflavin	each 1 mg.
(NH ₄) ₂ HC ₈ H ₅ O ₇	0.6 g.	<i>p</i> -Aminobenzoic acid; biotin	each 10 μ g.
Ascorbic acid	0.5 g.	Folic acid; vitamin B ₁₂	each 1 μ g.
Cysteine HCl	50 mg.		
Tween 80	1 g.		

Adjusted to pH 6.0-6.1 before addition of the required vitamins. Brought to final volume 1 l. with distilled water.

The pancreatic digest of vitamin-free casein (Table 1) was prepared according to Roberts & Snell (1946); it was used at a concentration of solids equivalent to 4 g./l. final medium. Both the acid-hydrolysed and pancreatic digests of casein were kept frozen until required.

Adenine, guanine, and uracil were dissolved in boiling distilled water acidulated with just sufficient HCl to effect solution. Xanthine was dissolved in boiling water, solution being aided by adding a minimum of 10% (w/v) NaOH solution. These solutions were made up to 0.5 mg./ml. and were kept at 5°.

Solids such as glucose, the phosphates, di-ammonium citrate, ascorbic acid and cysteine HCl were weighed out and added as needed for each fresh batch of medium. Sodium acetate was not added in this way because the casein digest solutions already contained acetate as a result of the prescribed adjustments of pH value with acetic acid and sodium or potassium hydroxide, and thus contributed a calculated 0.6% (w/v) of anhydrous sodium or potassium acetate to the final medium. Refrigerated Tween 80 was warmed and added directly as the final medium was being prepared.

The solution of mineral salts used in the medium (Table 1) was that of Ford *et al.* (1958).

The concentrations of vitamin solutions in 20% (v/v) ethanol in water were as follows: *p*-aminobenzoic acid, nicotinic acid, thiamine HCl, pyridoxal ethyl acetal HCl, Ca D-pantothenate, all 1 mg./ml.; riboflavin, 100 µg./ml. and vitamin B₁₂, 5 µg./ml. (each with 1 drop of concentrated HCl); DL-biotin and folic acid at 100 µg./ml. in 20% (v/v) ethanolic 1% (w/v) NaHCO₃. These solutions were kept at 5° in the dark and any necessary dilutions were made only when final media were prepared. Any excess of a diluted solution was discarded.

The media were dispensed in 10 ml. quantities into 6 × 5/8 in. rimless test tubes, capped with Oxoid aluminium caps, and heated in racks in the autoclave to 115°. Immediately on reaching this temperature the autoclave was turned off. Only fresh media were used and first inoculations were generally made immediately on cooling and never into media more than one day old.

Inoculation. Actively growing cultures passed through three successive transfers in MRS medium (de Man, Rogosa & Sharpe, 1960) were used. Inocula of 1 drop were taken directly from such cultures or from suspensions of organisms washed twice in saline. Both methods gave identical results. Three serial transfers were made into the test media.

Incubation and examination. Except for *Lactobacillus casei*, *L. plantarum*, *L. buchneri*, *L. cellobiosus*, *L. viridescens* and *L. brevis*, which were incubated at 30°, all tubes were incubated at 37° for about 18 hr. Visual observations of growth in each serial transfer were recorded and the optical density of the third serial transfer was also measured in tubes of 5/8 in. diameter in a Hilger Biochem Absorptiometer equipped with filter 61 (610 mµ).

RESULTS AND DISCUSSION

Descriptions of the *Lactobacillus* spp. are in general agreement in studies by Orla-Jensen (1919, 1943), Rogosa *et al.* (1953), Sharpe (1955), Wheeler (1955*a, b*) and Rogosa & Sharpe (1959). The cultural and biochemical characteristics of every strain included in the present work are known and have been repeatedly determined by one or often all of the above authors. Where group antisera were available

(Sharpe, 1955), the serological characteristics are also known. Therefore, except where it may be necessary in order to clarify certain relationships, detailed data about the general non-nutritional behaviour of the species will not be given. Certain general nutritional results, also, will not appear in the tables. For example, nicotinic acid and pantothenic acid were indispensable for the growth of all species. Exogenously supplied thiamine was required by all the heterofermentative species and by none which are homofermentative. These results are in entire agreement with those of Rogosa *et al.* (1953) and of Koser & Fisher (1950). Folic acid and *p*-aminobenzoic acid were not essential or stimulatory for any species. Biotin requirements were not investigated because of the nature of the basal medium (Table 1) required for the good growth of a variety of species.

The nutritional requirements of *Lactobacillus casei* and *L. plantarum* found were the same as recorded previously by Rogosa *et al.* (1953). All the variant strains of *L. casei* required added folic acid and pyridoxal whereas *L. plantarum* grew well in their absence. Rogosa *et al.* (1953) stated that vitamin B₆ (pyridoxal) was essential for the growth of some strains of *L. casei* and that there was 'severe limitation of growth in absence of added substrate' with others. The present results are therefore in excellent agreement with previous work, and because of the high correlation with other characteristics, one may differentiate with confidence between *L. casei* and *L. plantarum* on the basis of nutritional results combined with a few other data. The essential differentiating data are shown in Table 2. All strains of these species grew abundantly in the medium containing the full complement of vitamins; after incubation for 18 hr. the optical density values of the turbid cultures were generally at least 1.3 and often were 1.7 or more.

There are 8 known homofermentative species which cannot grow at relatively low temperatures (15°) and which nearly always grow at 45° or higher. These belong to the group designated *Thermobacterium* Orla-Jensen (1919, 1943). The detailed serological and physiological criteria by which they may be distinguished were described by Rogosa & Sharpe (1959). Until recently the distinction between *Thermobacterium bulgaricum* Orla-Jensen (1919, 1943), i.e. *Lactobacillus bulgaricus*, and *Thermobacterium jugurt* Orla-Jensen (1919, 1943) syn. *Lactobacillus jugurti* Rogosa & Sharpe (1959), was not clear. These species were confused by *Bergey's Manual* (1948, 1957), Rogosa *et al.* (1953), Wheeler (1955*a, b*) and Sharpe (1955). However, de Man (1956, 1960) isolated a sufficient number of strains of *L. bulgaricus* which conformed with Orla-Jensen's original description, thus enabling de Man to differentiate clearly between this species and *L. jugurti* and *L. helveticus*. Rogosa & Sharpe (1959), who used de Man's strains and some other isolates, confirmed entirely the results of de Man (1956, 1960) and Orla-Jensen (1919, 1943). A study of the vitamin requirements of these organisms has now shown that a similar differentiation is also possible by this method; pyridoxal is essential for the growth of *L. jugurti* and *L. helveticus* but not for *L. bulgaricus*.

Lactobacillus helveticus differs from *L. jugurti* only in fermenting maltose (Orla-Jensen, 1919, 1943) and dextrin (Wheeler, 1955*a*); but similar or the same enzymes are involved in the fermentations of these α -D-glucosides. The organisms are serologically identical, and produce distinctively large amounts of DL-lactic acid. The nutritional data support the view that these organisms are closely related species, since both organisms have identical requirements. There may be some

Table 2. *Differentiating characters of the known species of the subgenus Streptobacterium (Orla-Jensen)*

<i>Lactobacillus</i> spp.	Serological group	Molecular configuration of lactic acid	Gas from citrate	Fermentation of					Requirement for		
				Arabinose	Lactose	Melibiose	Raffinose	Rhamnose	Riboflavin	Pyridoxal	Folic acid
<i>L. plantarum</i>	D	DL	-	±*	+	+	+	- or weak	±†	-	-
<i>L. casei</i> var. <i>casei</i>	B, C	L(+)	+	-	+	-	-	-	+	+ or stimulatory	+
<i>L. casei</i> var. <i>rhamnosus</i>	C	L(+)	+	-	+	-	-	+	+	+ or stimulatory	+
<i>L. casei</i> var. <i>alactosus</i>	B, C	L(+)	+	-	-	-	-	-	+	+ or stimulatory	+

* 69 % of strains in the United States of America (Rogosa *et al.* 1953) and 79 % of strains in the United Kingdom (Wheater, 1955*b*).

† Rogosa *et al.* (1953) described two varieties of *L. plantarum*, one of which had a requirement for riboflavin correlated with other differences. They may be different even in a species sense.

merit in the proposal of de Man (1956) to unite these species under the name *L. helveticus* emend.

Lactobacillus bulgaricus and *L. lactis* possess some common features, particularly the same group antigen and the ability to produce equivalent amounts of D(–) lactic acid in milk; but *L. lactis* differs in fermenting maltose, sucrose and salicin (Rogosa & Sharpe, 1959). *L. lactis* grew profusely, whereas *L. bulgaricus* grew poorly or not at all in the complete medium (Table 1). Under these circumstances, when poor delayed growth occurred with *L. bulgaricus*, erratic and non-reproducible requirements were exhibited. Modifications of the medium in Table 1, such as the addition of lactose, pyridoxal phosphate, riboflavin phosphate and pantothenic acid improved growth. But the media were still unsatisfactory for *L. bulgaricus*; growth was poor and unpredictable from small inocula and sometimes tended to improve with each serial transfer. It was obvious that adaptation to unfavourable conditions was taking place with time. When the strains of *L. bulgaricus* were eventually induced to grow through repeated subcultivation, most of the erratic requirements disappeared and the single riboflavin requirement resembled that of *L. lactis* (Table 3). Folic acid at 10 µg./l. completely prevented the growth of many strains of *L. bulgaricus* and it was necessary to decrease the folic acid content to 1 µg./l. or to eliminate it entirely. Folinic acid at 10 µg./l. also was toxic. These difficulties were not encountered with *L. lactis* in the medium of Table 1; further work on the nutrition of *L. bulgaricus* is required.

Lactobacillus lactis differs from *L. acidophilus*, generally, in the quantity of acid produced in milk and in the different configurations of the lactic acids produced. *L. acidophilus* ferments amygdalin and cellobiose and quenches the fluorescence of aesculin, while *L. lactis* does not (Rogosa & Sharpe, 1959). The vitamin nutrition of these organisms is also different: *L. acidophilus* exhibited a requirement for folic acid which was not shown by *L. lactis* (Table 3).

Table 3. *Some nutritional requirements of the subgenus Thermobacterium Orla-Jensen*

<i>Lactobacillus</i> spp.	Riboflavin	Pyridoxal	Folic acid	Vitamin B ₁₂	Thymidine
<i>L. helveticus</i>	+	+	–	–	–
<i>L. jugurti</i>	+	+	–	–	–
<i>L. bulgaricus</i>	+	–	–	–	–
<i>L. lactis</i>	+	–	–	–*	–
<i>L. acidophilus</i>	+	–	+	–*	–
<i>L. leichmannii</i>	–	–	+	+ or stimulatory†	–
<i>L. delbrueckii</i>	+	–	–	–	+
<i>L. salivarius</i>	+	–	+	–	–

+ = requirement; – = no exogenous requirement.

* Some strains exhibit erratic requirements under highly aerobic conditions, particularly in the absence of certain deoxyribosides.

† In absence of deoxyribosides.

On the basis of fermentation tests alone it may be difficult to distinguish between *Lactobacillus acidophilus* and *L. leichmannii*, the only difference being the fermentation of galactose by the former but not by the latter. Apart from this property,

and the production of DL-lactic acid by *L. acidophilus* and D(−) lactic acid by *L. leichmannii* (a determination which may not be routinely easy for some laboratories), the only other consistently unambiguous differentiating property is the formation of acid in milk by *L. acidophilus*. However, all the strains of *L. acidophilus* tested required riboflavin whereas *L. leichmannii* did not (Table 3). Thus, an organism which exhibits the characters described by Rogosa & Sharpe (1959) could be correctly placed in one or other of these species by a titration of the acidity produced in milk and by the presence or absence of a riboflavin requirement.

Lactobacillus delbrueckii and *L. leichmannii*, although alike in producing D(−) lactic acid in suitable media and in not fermenting lactose in milk, are distinctly different in their action on aesculin and in the fermentation of cellobiose, salicin and trehalose (Rogosa & Sharpe, 1959). They also exhibited consistent differences in their nutritional behaviour. Whereas *L. delbrueckii* required riboflavin + thymidine, but not folic acid or vitamin B₁₂, *L. leichmannii* required folic acid + vitamin B₁₂ but not riboflavin or thymidine (Table 3). It would be desirable to have information about more than the four available strains of *L. delbrueckii*; unfortunately strains so named and received from other workers very often proved to be members of other species. This small number of strains within a species is unusual since many species have been represented by a large number of strains, sometimes hundreds, in repeated studies by the present authors and others.

Lactobacillus salivarius (Rogosa *et al.* 1953) possesses a new group antigen and is otherwise distinct from other species. Although the vitamin requirements of *L. acidophilus* were the same as those of *L. salivarius*, these species are consistently different morphologically, serologically, in the fermentation of cellobiose, mannitol and sorbitol, and usually behave differently in melibiose, raffinose, rhamnose and salicin (Rogosa & Sharpe, 1959).

The status of the heterofermentative species of the genus *Lactobacillus* is more uncertain and may reflect a higher degree of variability. The general characteristics of *L. fermenti*, *L. buchneri*, *L. brevis*, *L. cellobiosus* and *L. viridescens* have been described either by Orla-Jensen (1919, 1943), or in *Bergey's Manual* (1948, 1957), or by Rogosa *et al.* (1953) and Rogosa & Sharpe (1959). Of these species, only *L. fermenti* has the Group F antigen (Sharpe, 1955), grows at 45° and not at 15°, and typical strains do not ferment arabinose (79%) and xylose (77%).

Lactobacillus brevis and *L. buchneri* are related antigenically but also, peculiarly, have the same group antigen as *L. lactis* (Sharpe, 1955). Eighty-eight% of the *L. brevis* strains fermented arabinose and xylose, whereas all the strains of *L. buchneri* fermented arabinose, with only 12% fermenting xylose. Nearly all strains of both species (88–100% in different tests) fermented α -methyl-D-glucoside; but with many other carbohydrates there seems to be even greater variation in the behaviour of individual strains (Rogosa & Sharpe, 1959). However, despite these disconcerting variations, *L. buchneri* and *L. brevis* can be differentiated by means of the information already given and that shown in Table 4. In our experience with several hundred strains of *L. brevis* and *L. buchneri*, the former has consistently required folic acid for growth and has not fermented melezitose, while *L. buchneri* has not required folic acid and has fermented melezitose.

Lactobacillus cellobiosus may be recognized from the descriptions given by Rogosa *et al.* (1953) and Rogosa & Sharpe (1959), and by the information given in Table 4.

Table 4. *Principal differentiating characters of heterofermentative species of the genus Lactobacillus*

<i>Lactobacillus</i> spp.	Growth		Serological group	Fermentation of		Requirement for		
	15°	45°		Cellobiose	Melezitose	Riboflavin	Pyridoxal	Folic acid
<i>L. fermenti</i>	—	+	F	—	—	—	—	—
<i>L. buchneri</i>	+	—	E	—	+	±*	—	—
<i>L. brevis</i>	+	—	E	—	—	—	—	+
<i>L. cellobiosus</i>	slight or —	—	not known	+	—	—	—	—
<i>L. viridescens</i>	+	—	not known	—	—	+	stimulatory	stimulatory

* ± = 75 % of strains exhibit requirement.

L. viridescens (Niven & Evans, 1957) was further described by Rogosa & Sharpe (1959) and the vitamin requirements of Table 4 conform to the original description.

With most of the organisms the vitamin requirements were sharp and unequivocal and in the absence of a required vitamin there was generally slight or no growth even in the first serial subculture. It was of particular interest that, in instances where only tentative or presumptive results had been obtained by other methods, predictions of the species identities from the nutritional data were made with a high degree of accuracy. Also, with the species, which were capable of growing well in any of the non-deficient media used, the results were in highly satisfactory agreement with those obtained some years previously. Very often, especially in examinations of a large number of isolates from similar sources, certain strains are encountered which are alike but do not conform exactly to the characteristics of any recognized species. For example, in recent work on lactobacilli from silage, Keddie (1959) isolated a number of strains which differed from known strains of *Lactobacillus plantarum* in consistently failing to ferment structurally dissimilar carbohydrates such as maltose and mannitol. The strains clearly belonged to the subgenus *Streptobacterium* Orla-Jensen and 'resembled' *L. plantarum* more closely than *L. casei*. Consequently Keddie concluded that they should be attached to the former species. Later, when representative strains kindly supplied by Dr R. M. Keddie were examined by us for their nutritional requirements it was found that they had heterogeneous requirements including, in some instances, a requirement for folic acid not previously found for any *L. plantarum* isolate. The differences in nutritional behaviour among his strains suggest that these strains are not *L. plantarum* but rather comprise a new species. It has been our repeated experience that the nutritional data often serve to emphasize the heterogeneity of certain groupings where different species have been grouped together.

We do not suggest immutability and there are well known instances whereby media and experimental conditions may influence nutritional requirements or growth antagonisms. There is the example described by Ford *et al.* (1958) in which *Streptococcus bovis* exhibited a number of nutritional requirements when cultivated aerobically, although anaerobically the organism grew well in the absence of any added vitamin. The interrelation between pyridoxine and alanine was noted by Snell & Guirard (1943), and Griffin & Racker (1955) showed that the CO₂ requirement of *Neisseria gonorrhoeae* could be replaced by hypoxanthine, uracil and oxalacetate. These are only a few of the examples which can be cited; they serve to emphasize the caution which must be exercised. It is advisable not to generalize beyond the restrictions of specific experimental conditions, and in determining the optimal conditions for the growth of a significant number of strains of representative species. From this viewpoint, nutritional studies have been very helpful in the taxonomic study of the lactobacilli and have sometimes disclosed problems for further study, such as the unknown mechanism of inhibition of the growth of *Lactobacillus bulgaricus* by folic acid.

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The Air Spora of a Cowshed

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SUMMARY

Study of the air spora of a cowshed by means of a Hirst Automatic Volumetric Spore Trap showed an atmospheric concentration of fungal spores ranging from 95,000 to 16,000,000 spores/m³. There was a direct relationship between the hours during which hay was being fed and the highest concentrations of spores. *Aspergillus-Penicillium* and *Mucor* types of spore were predominant, and hyphal fragments including conidiophores were the third most numerous component. The findings are discussed with reference to human and animal fungal disease.

INTRODUCTION

Although progress has been made in the study of the outdoor air spora there is little information on the air spora within buildings (see Maunsell, 1954; Gregory, Hirst & Last, 1953; Richards, 1954). Ainsworth (see Fuller, 1953) in a short series of Petri dish exposures found the fungus spore concentration in cowsheds to be 10-100 times that of the farmyard air. Rolle & Kolb (1954) using a similar method obtained *Absidia ramosa*, *Mucor pusillus*, *Aspergillus fumigatus*, *A. flavus*, *A. niger*, *A. glaucus* (series), and *Scopulariopsis brevicaulis* in abundance in farm buildings. They also isolated most of these species from adjacent samples of hay and from the saliva of the housed cattle. Ainsworth & Austwick (1955*a, b*) confirmed that these species (and especially *Absidia ramosa* and *Aspergillus fumigatus*) were constantly associated with farm animals and could be isolated from almost every bovine and equine skin scraping received for examination as suspected ringworm. These few records of fungi from the environment of farm animals clearly show that the species present are just those responsible for the commoner systemic mycoses of man and animals in Britain. Aspergillosis in birds and mammals, mucormycosis in cattle, pigs and man, and bovine mycotic abortion are all associated with these fungi in their pathogenic state, yet the spores of these potential pathogens are normally present in the atmosphere of farm buildings. A key to the sporadic occurrence of these mycoses may be found in the quantitative and qualitative comparison of the air spora of cowsheds where animals are housed under normal conditions with those premises in which mycoses such as mycotic abortion have occurred. The preliminary study of the air spora of a cowshed reported here was carried out at the Ministry of Agriculture, Fisheries and Food, Central Veterinary Laboratory, Weybridge, Surrey, England, during November and December 1956.

METHODS

Building. The cowshed was a modern brick and concrete building with a concrete floor, 98.0 m. long, 11.4 m. wide, 5.0 m. to the top of the sloping roof and 2.0 m. to the top of the side walls. The cubic capacity was thus about 750 m.³ (Fig. 1). The number of milking cows housed was 24. The hay in use for feeding during the period of the experiment was of fair quality.

Sampling. A Hirst Automatic Volumetric Spore Trap (AVST) mounted on a tripod, bringing the aperture 0.5 m. from the floor, was used for sampling. It was sited approximately in the centre of the north access passage of the outside of the stalls. The aperture was facing and 0.5 m. away from the manger which was of concrete construction and had an outside wall 1 m. high (see Fig. 1). In this way the trap was within 2 m. of the head of the nearest animal. The pump was operated from a mains electricity supply.

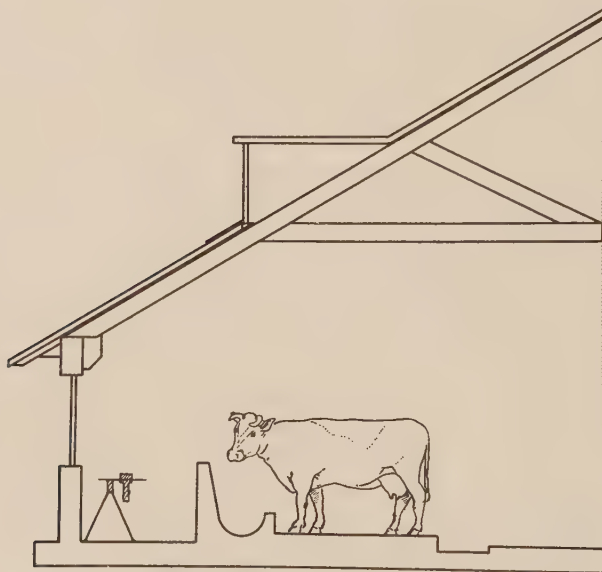


Fig. 1. Diagram of position of spore trap in cowshed.

The rate of flow was adjusted to approximately 10.0 l./min. and keyed 'Vaseline'-coated slides were used for the trace (Hirst, 1953). The slide moved at 2 mm./hr. and the 24 hr. trace was thus 48 mm. long. Slides were changed at 17.00 hr. daily and the trap was operated continuously for periods of 4 days at a time. The two periods selected for analysis were 12-16 and 19-23 November 1956 inclusive. The weather during the first period was damp and mild with dense fog on the night of 13 November; during the second period it was fine and cold with a severe frost on the night of 22 November.

Scanning. After mounting in 'Solvar'-lactophenol scanning was carried out by means of short or cross traverses (Hirst, 1953). At certain periods of the day so many spores were being caught that the deposit was too thick for scanning, and these periods were therefore sampled separately on 18 December by using

0.5–2.5 min. exposures from which the spore content at these times could be effectively calculated.

Classification of spores. The spores were classified into seven easily recognizable groups with a group for hyphal fragments, chiefly conidiophores.

Methods for the calculation of spore content. From the rate of movement of the slide in the trap, the volume of air drawn into the trap and the area of trace scanned in counting, the number of spores/m.³ air at different times of the day could be estimated.

Operations in the cowshed. Operations carried out in the cowshed appeared to be largely responsible for the changes in the concentration of spores in the air; a summary of the routine procedures is given in Table 1. The time of each operation varied slightly but the amount was not more than ± 0.5 hr.

Table 1. *Daily operations in cowshed*

Time	Operation
07.00	Bedding straw and dung cleared out. Cows washed down
08.00	Cake fed and cows milked
09.15	Kale and hay fed
10.15	Cows groomed and turned out to pasture
10.30	Dung cleared out and mangers cleaned, forestall swept and cowshed hosed out
13.30	Kale and hay (in bales) brought in to access passages and bedding straw put down
14.45	Cows brought in from pasture and washed down
15.15	Cake fed and cows milked
15.30	Hay and kale fed, cows bedded up and floor washed down
16.00	Cowshed closed

Supplementary methods of sampling. In addition to the visual sampling with the automatic volumetric suction trap two cultural methods were also used. In one suggested by Professor A. J. P. Oort (University of Wageningen) and used in conjunction with the suction trap, the slides were tightly covered with a 2.5 cm. wide strip of washed semi-transparent plastic film ('Polythene') which was then smeared lightly with a commercial pectic jelly prepared from apple ('appelstroop'). Slides so prepared were then exposed in the suction trap and after exposure the plastic was carefully removed and cut up transversely into four strips 12 mm. wide. These were placed in 50 ml. sterile distilled water, shaken to produce an even suspension, and then dilutions were mixed with molten 2 % malt agar in Petri dishes. The second supplementary method of sampling was by the exposure of 10 cm. Petri dishes containing 2 % malt agar to gravity sampling for periods varying from 15 sec. to 5 min. on a table 1 m. above the floor of the cowshed.

RESULTS

Visual results with the AVST

The first 4-day period of operation revealed that even with fair quality hay the cowshed air spora was very much heavier than had been imagined, and that the peak periods of spore deposition corresponded to the times at which the hay was handled. These periods occurred just after milking in the morning, in the early afternoon, and again after milking in mid-afternoon. Because the traces at these times were

too thick for scanning and separate sampling had been undertaken for the peak periods, it has been necessary to divide the observations into peak and off-peak periods, the latter alone being based on continuous sampling. The daily off-peak means are thus calculated over nine 2-hourly observations on 8 days and the peak period means are based on two traces obtained within 5 min. of each other at 16.45 hr. on 18 December. The data on the atmospheric spore content of the cowshed are summarized in Table 2.

Table 2. Concentrations of spores in cowshed air

Figures are for 8-day readings.

Spore type	Spore content thousands/m. ³						
	Off-peak				Peak average	Total percentage	
	Daily average	Hourly		Off-peak		Peak	
		Highest	Lowest				
Aspergillus*	32.6	500.0	1.3	12,390	34.2	75.1	
Mucor type	33.0	144.0	1.0	1,217	34.6	7.4	
Cladosporium	6.9	86.0	0.7	1,570	7.2	9.5	
Ustilago	0.3	2.2	0.0	168	0.3	1.0	
Monotospora	1.0	12.0	0.0	69	1.0	0.4	
Hyphae†	16.9	60.2	0.0	851	17.7	5.1	
Streptomyces‡	.	5.0	0.0	.	.	.	
Non-classified	4.5	.	.	232	4.7	1.4	
Totals:	95.2	.	.	16,497	100.0	100.0	

* Including Penicillium. † 4 days only. ‡ 6 days only.

Total spore concentrations. From the colour and thickness of the spore deposit on the exposed slides (Fig. 2), it was not surprising that the total of spores caught was in excess of any figure previously reported from the AVST. Gregory *et al.* (1953) recorded the highest concentration of spores with 360,000/m.³ in the cellar of an

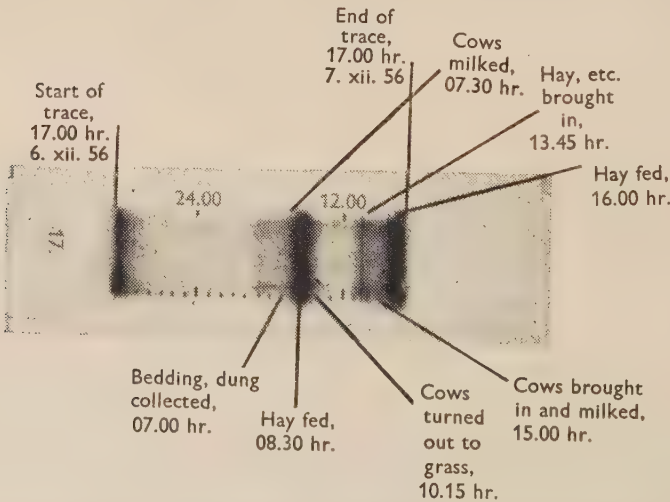


Fig. 2. Slide exposed from 17.00 hr. from 6 December to 17.00 hr. 7 December 1956.

old country mansion with large fruiting bodies on *Merulius lacrymans* a short distance from the trap. The normal level of spores in the outdoor air at 2 m. above the ground level rarely rises above 100,000 spores/m.³ (Hirst, 1953), although Sreeramulu (1958) reported over a million spores/m.³. The daily off-peak average spore content recorded in our observations was 95,000/m.³; and very large day-to-day fluctuations were found amongst the individual spore types. On some days over 400 times as many spores of one type were present as on other days. In the peak periods the total spore concentrations reached 16,500,000/m.³.

Diurnal periodicity. Unfortunately not enough reliable data were available for analysis of the diurnal periodicity of the spore types and the only comparisons to be drawn are those between peak and off-peak periods. Day-to-day variation of the spore content was very great. In Table 3 the total concentrations at 01.00 hr. are given for 4 consecutive days (20–23 November). These show that the numbers of mucoraceous spores at times exceeded the combined total of the other types and that in off-peak periods the spore content of the air varied considerably at the same time each day.

Table 3. *Spore content at 01.00 hr. on 4 consecutive days*

Spore type	Spore content thousands/m. ³ at 01.00 hr. on 4 consecutive days			
	Date during November 1956			
	20	21	22	23
Aspergillus	6.6	13.1	52.1	12.7
Mucor type	46.9	142.6	31.8	10.2
Cladosporium	5.3	3.9	7.1	3.0
Ustilago	0.6	0.0	0.0	0.0
Monotospora	1.2	0.0	0.0	0.0
Hyphae	9.8	12.5	17.1	15.5
Streptomyces	0.7	2.3	0.0	0.0
Non-classified	6.0	2.2	1.3	0.3
Totals	77.1	181.6	109.4	42.7

Spore types

The spore types found in the trace did not appear to differ greatly from those found in the outdoor air; only two components, the hyphal fragments and the Monotospora-type spore, were new records. The main interest, however, is that the proportions of the different classes were almost reversed, with Mucor and Aspergillus-type spores becoming the predominant forms (combining to give 70 % off-peak and 85 % during peak) and Cladosporium falling to third or fourth place (7 and 9 %, respectively).

Mucor type. The characteristic sporangiospores of *Mucor* and *Absidia* spp. were noticeably unevenly distributed on the traces, apparently due to the fact that a great many had passed through the aperture of the trap within their sporangia and these bodies had burst on striking the surface of the slide. Many such apparently burst sporangia with the characteristic pear-shaped columellae of *Absidia* were seen.

Aspergillus type. Although this type of phialospore covers both *Aspergillus* and

Penicillium it is believed that the latter is rarer in hay samples and that *aspergilli* predominate.

Cladosporium type. No great diurnal variation in the numbers of *Cladosporium* spores which might have corresponded to that seen during the late summer in outdoor air was noted. The highest concentration was above the values previously recorded and it seems that the spores were not derived from the outdoor air. Comparison with the figures of Hirst (1953) shows that *Cladosporium* spores are only likely to be found in low numbers by the end of October.

Monotospora. *M. lanuginosa* is one of the commonest fungi found in hay especially when it has been heated during maturation, for it has an optimum growth temperature of 45–50° (Rehe, 1927). The spores are globose, about 10 μ . diam., with thick dark-brown warted walls. This type does not seem to have been reported before as a component of the air spora, but it may have been overlooked because of its close resemblance to a small *Epicoccum* spore.

Ustilago. Smut spores varied considerably in numbers but were never very abundant with a maximum 2-hourly concentration of 2000/m.³ in an off-peak time. On the last day of sampling, corresponding to an outside heavy air frost, no smut spores were seen.

Streptomyces. Only incomplete figures for the aerial spores of these actinomycetes were obtained, but they are sufficient to indicate that streptomycete spores were a characteristic and probably important part of the cowshed air spora.

Hyphae. Vegetative hyphal fragments and conidiophores are rarely classified as a component of the outdoor air spora, but the figures available from our observations show them to be third in numerical order in off-peak periods and fourth in the peaks. They were very variable and could not always be attributed to a particular fungus. Some pieces were clearly the sporangiophores of mucoraceous fungi, bearing columellae, others were dematiaceous conidiophores, and the sporing heads of *aspergilli* were also frequent.

Non-classified spores. This category included a number of readily identified as *Alternaria*, *Helminthosporium*, *Epicoccum*, *Ganoderma* and other coloured basidio-spores and rust uredospores, together with a number of unidentified spores. None of these categories was present in sufficient numbers for separate classification.

Results from culture methods

Pectic jelly AVST slides. With the methods described above, the dilution plating was carried out on 17–19 December, with 1/50 dilutions (Table 4). Probably all that can be said is that the method appears to be promising.

Petri plate exposures. In a single estimation by exposing a Petri dish for 15 sec. 90 colonies of fungi were obtained. With longer exposures the number of colonies was too great to be counted.

The species of fungi recovered in culture by these two methods were not studied in detail but the following were recorded: **Absidia corymbifera* (Cohn) Sacc. & Trot., **A. ramosa* (Lindt) Lendner, *Mucor racemosus* Fresen., **M. pusillus* Lindt, **Rhizopus arrhizus* Fischer, **Aspergillus flavus* Link, **A. fumigatus* Fresen., **A. nidulans* (Eidam) Wint., **A. niger* van Tieghem, *A. ochraceus* Wilhelm, *A. glaucus* series, **A. terreus* Thom, *Trichoderma viride* Fr., *Cladosporium herbarum* Fr., *Penicillium piceum* Raper & Fennell, *Chaetomium globosum* Fr.

Most of these species are fast growing, and on plates they completely obscured any slow growing fungi. Those organisms marked * are potentially pathogenic to animals.

Table 4. *Numbers of colonies on dilution plates from pectic jelly slides*

Portion of trace, 17-18 Dec. 1956	Numbers of colonies/plate. Dilutions				Estimated equivalent, spores/m. ³
	1/50	1/2,500	1/125,000	1/6,250,000	
17.00-23.00 hr.	260	7	—	2	4,194
23.00-05.00 hr.	480	74	4	3	6,944
05.00-11.00 hr.	*	440	42	1	350,000
11.00-17.00 hr.	*	*	104	30	3,610,000

* Colonies too numerous to count.

DISCUSSION

The most interesting result of this aerobiological study seems to be the very high density of airborne spores encountered in an ordinary cowshed. The spore content of the air was high throughout the 24 hr., and an exceptionally heavy spore load followed the disturbance of the hay, indicating that the 'dust' raised in cowsheds may be composed chiefly of fungus spores and hyphae. Very few plant, animal or mineral fragments were seen on the slides from the AVST.

Of the different spore types observed the *Aspergillus*-*Penicillium* type and the *Mucor* type are usually of minor importance in outdoor air spora sampled with the AVST and have never before been reported in such large numbers, although they may appear in some numbers on gravity plate cultures. These groups have been shown to increase in numbers in air in winter (Richards, 1954; Vallery-Radot, Halpern, Secretain & Domart, 1950), possibly associated with the feeding of farm livestock with hay. It seems certain that small local concentrations of spores must be set up every time a hay or straw stack is disturbed. The high number of hyphal fragments is also a feature not previously reported and may show that the spores are released into the air with sufficient friction to break off the complete sporophore from its substrate when the hay is pulled apart and placed in the mangers.

The virtual absence of several of the more prominent spore types found in the outdoor air, such as ascospores, basidiospores, and *Erysiphe* conidia is perhaps satisfactorily explained by the general diminution of the outdoor air spora in winter, the density of the deposit on the slides and the dryness characteristic of the cowshed. The two newly reported and easily recognized categories of airborne spores are the *Streptomyces*- and the *Monotospora*-types. The former may be the source of many of the streptomycete colonies frequently occurring as laboratory contaminants, while the latter seems to be an 'indicator species' for over-heated hay, as it is rare in good quality samples.

When the pathogenic significance of the fungi found in the cowshed air is considered it is necessary to deal with individual species of fungi. In the list given above those species marked with an asterisk (*) are potentially pathogenic to men or animals and as these represent the majority of the *Aspergillus* and mucoraceous colonies obtained, most of the spores classified visually in these two categories belonged to these species. The inhalation of *A. fumigatus* spores by animals can produce pulmonary infection and the ingestion of mucoraceous spores is thought to

be one infection route for mucormycosis of the alimentary tract in animals, e.g. guinea-pigs (Ainsworth & Austwick, 1955*a*). The possibility of an initial respiratory infection leading to mycotic abortion in cattle was discussed by Bendixen & Plum (1929) and the normal spore concentrations in the atmosphere of a cowshed obtained during the present study may provide a useful basis for investigating the little known epidemiology of these mycoses.

The possible value of these findings in the medical field is probably not so much concerned with the direct pathogenicity of the fungi as with their ability to act as allergens. Hyde, Richards & Williams (1956) showed how the spores of certain species of fungi produce allergic respiratory symptoms, e.g. asthma, in subjects not normally exposed to concentrations of spores greater than are found in the outdoor air. Ill-defined respiratory trouble associated with the handling of mouldy hay and straw often referred to as 'farmer's lung' (Fuller, 1953; Frank, 1958) has yet to be related to a specific cause, but with spore concentrations in air as high as those recorded in this work it should be possible to locate one or more sensitizing agents among the many different components present in cowshed air.

Estimated spore intake of cattle and man. According to Hall & Brody (1933) a standing cow has the following air intake (subject to a 20 % coefficient of variation): *Tidal air* (volume inhaled at one inspiration), 3800 ml.; *respiration rate*, 27/min.; *minute volume* (volume of air inspired/min.), 100 l. From these data it may be calculated that in 10 min. a standing cow respires from about 1 m.³ air, or from 6 m.³/hr.

If a continuous spore load of 100,000 spores/m.³ be assumed, then the spore intake of each cow over the 19 hr. she is in the cowshed (not of course standing for the whole time) is of the order of 600,000 spores/hr. or 11,400,000 spores daily. As there are two peak periods involved each with a possible duration of 1–2 hr., one 16 hr. exposure at 100,000 spores/m.³ and one of 3 hr. at 16,000,000 spores/m.³ would give a total intake during the housed hours of the order of 297,600,000 spores.

In man the tidal air is about 500 ml., the respiration rate 15–20 and the minute volume 10 l., so that the intake of spores may be taken direct from the figures obtained from the AVST (Tables 2 and 3). Total exposure, however, would differ considerably, but in this case would include breathing for some time in each of the three peak periods during the day.

Thanks are offered to Dr A. W. Stableforth and Dr G. Slavin for allowing me to undertake the sampling in a cowshed housing an experimental herd at Weybridge and to Mrs J. D. Cartner for her technical assistance. Acknowledgement is also made to the Administrators of the Colombo Plan for the award of a fellowship which enabled the work to be undertaken. Thanks are also offered to Mr P. K. C. Austwick of Weybridge for guidance, and to Professor P. H. Gregory for facilities for this work in the Department of Botany, Imperial College of Science and Technology, London, S.W. 7.

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